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## Description

The present invention relates to new gram-positive expression control DNA sequences, to expression vectors containing these DNA sequences, to host cells transformed with these expression vectors and to methods for producing pro- and eukaryotic proteins by using the new expression control DNA sequences, vectors and transformants.

Most recombinant DNA work to date has been carried out with *Escherichia coli* (*E. coli*). *E. coli* is a member of the gram-negative class of bacteria which contain two layers of membranes enclosing a periplasmic space. Many of the products produced in *E. coli* are secreted into this periplasmic space, if secreted at all. Few products are secreted outside the living cells into the growth media.

On the other hand, *Bacillus subtilis* (*B. subtilis*) is a member of the gram-positive class of bacteria which contain only a single layer of bacterial membrane. Thus *B. subtilis* can produce large amounts of protein which are secreted directly into the growth medium. Moreover, production of proteins in *B. subtilis* is advantageous since the organism is non-pathogenic and does not produce endotoxins. In addition, *B. subtilis* has been extensively studied and is the archetype for genetic studies among gram-positive microorganisms.

Although the general approach to gene cloning in *E. coli* is applicable to *B. subtilis*, attempts to produce a useful product of heterologous gene cloned into *B. subtilis* and secreted into the growth media have been retarded and made especially difficult because of the general lack of suitable cloning and expression vectors. This paucity of expression vectors is explained in part by the lack of recognition of foreign transcription and translation initiation signals in *B. subtilis*. Consequently, the well known *trp* (Hallewell, R.A. and S. Emtage, *Gene* 9, 27-47 [1980]), *lac* (K. Itakura et al., *Science* 198, 1056-1063 [1977]; Roberts, T.M. et al., *Proc. Nat. Acad. Sci. USA* 76, 5596-5600 [1979]), *lpp* (Lee, N. et al., *J. Bacteriol.* 146, 861-866 [1981]; Zwiebel, L.J. et al., *J. Bacteriol.* 145, 654-656 [1981] and Natamura, K. and M. Inouye, *Cell* 18, 1109 [1979]) and bacteriophage  $\lambda$  *P<sub>L</sub>* (Bernard, H. et al., *Gene* 5, 59-76 [1979]) transcription and translation-directing systems are not functional in *B. subtilis*. Thus, with the exception of mouse dihydrofolate reductase (Grange et al. *Nucleic Acids Research* 12, 3585-3601 [1984] and a few drug resistance genes from gram-positive organisms such as *staphylococcus* and *streptococcus*, few foreign genes encoding prokaryotic and eukaryotic proteins have been expressed in *Bacillus*, especially *B. subtilis*, (for review see "Genetics and Biotechnology of Bacilli", eds. A.T. Ganesan and J.A. Hoch; Academic Press, Inc. [1984] and dissertation of J. Palva, infra). Moreover, the expression yield is in general small, and therefore the development of superior expression vectors having potent promoters for *Bacillus subtilis* has been desired.

At present, the known *Bacillus subtilis* promoters with the respective base sequences clarified include the *veg* promoter, *trns* promoter, *pen P* promoter (C.P. Moran Jr. et al., *Mol. Gen. Genetics* 186, 339-346 [1982]), *spo VC* promoter (C.P. Moran Jr. et al. *Nucl. Acids Res.* 9, 5979-5990 [1981]), *spo VG* promoter (C.P. Moran Jr. et al., *Cell* 25, 783-791 [1981]),  $\sigma$  29 G3a promoter,  $\sigma$  29 G3b promoter,  $\sigma$  29 G2 promoter,  $\sigma$  29 A1 promoter (C.L. Murray and J.C. Rabinowitz, *J. Biol. Chem.* 257, 1053-1062 [1982]), *pMG 102* promoter (M.Z. Gilman et al., *Nucl. Acids Res.* 9, 5991-6000 [1981]), *spo 1-15* promoter (G. Lee et al., *J. Mol. Biol.* 139, 407-422 [1980]), *spo 1-16* promoter (G. Lee et al., *Molec. Gen. Genetics* 180, 57-65 [1980]), and *SPO2* promoter (R.G. Schoner et al., *Gene* 22, 47-57 [1983]). Among them, the *SPO2* promoter (R.G. Schoner et al., supra) and the *veg* promoter (European patent application, publication no. 116411) are the only promoters that have actually been utilized in gene expression.

Under these circumstances, it is thus of advantage to develop more potent gene expression systems for use in gram-positive bacteria, e.g. *Bacillus*, particularly *B. subtilis*. In this respect, the versatile expression vectors of the present invention are particularly important because they allow for the first time the expression of genes encoding prokaryotic and eukaryotic proteins in *Bacillus*, especially *B. subtilis*, and other gram-positive host cells under the control of transcription initiation and termination DNA-sequences of gram-negative origin.

The present invention specifically provides gram-positive bacterial expression control DNA sequences having proximal to one end a transcription initiation DNA sequence of gram-negative bacterial origin, proximal to the other end a transcription termination DNA sequence of gram-negative or gram-positive bacterial origin, and having intermediate said transcription initiation DNA sequence and transcription termination DNA sequence a ribosome binding site-encoding DNA sequence of gram-positive or gram-negative origin optionally operatively linked to a foreign gene encoding prokaryotic or eukaryotic polypeptides as well as a process for the manufacture of such expression control DNA sequences, which process comprises combining in the downstream direction (5' to 3') a transcription initiation DNA sequence of gram-negative bacterial origin, a ribosome binding site-encoding DNA sequence of gram-positive or gram-negative origin, and a transcription termination DNA sequence of gram-negative or gram-positive bacterial

origin to a functional unit by techniques of DNA recombination well-known in the art.

More precisely, the present invention allows the following combinations of: (a) a transcription initiation DNA sequence (promoter) of gram-negative bacterial origin with a ribosome binding site-encoding DNA sequence of gram-positive bacterial origin and a transcription termination DNA sequence of gram-negative bacterial origin,

(b) a transcription initiation DNA sequence (promoter) of gram-negative bacterial origin with a ribosome binding site-encoding DNA sequence of gram-negative bacterial origin and a transcription termination DNA sequence of gram-negative bacterial origin,

(c) a transcription initiation DNA sequence (promoter) of gram-negative bacterial origin with a ribosome binding site-encoding DNA sequence of gram-positive bacterial origin and a transcription termination DNA sequence of gram-positive bacterial origin, and

(d) a transcription initiation DNA sequence (promoter) of gram-negative bacterial origin with a ribosome binding site-encoding DNA sequence of gram-negative bacterial origin and a transcription termination DNA sequence of gram-positive bacterial origin.

The term bacterial origin used in connection with transcription initiation DNA sequences comprises (a) naturally occurring bacterial transcription initiation sequences and functional variations thereof including substitutions or inversions of single or several nucleotides and repeats of such transcription initiation DNA sequences and (b) chemically synthesized (synthetic) transcription DNA sequences capable of initiating transcription in bacteria.

The term bacterial origin used in connection with ribosome binding site-encoding DNA sequences comprises (a) naturally occurring bacterial ribosome binding site-encoding DNA sequences and functional variations thereof including substitutions or inversions of single or several nucleotides and (b) chemically synthesized (synthetic) ribosome binding site-encoding DNA sequences capable of initiating translation in bacteria.

The term bacterial origin used in connection with transcription termination DNA sequences comprises (a) naturally occurring bacterial transcription termination DNA sequences and functional variations thereof including substitutions or inversions of single or several nucleotides and repeats of such transcription termination DNA sequences and (b) chemically synthesized (synthetic) transcription termination DNA sequences capable of terminating transcription in bacteria.

In a preferred application genes encoding prokaryotic or eukaryotic proteins can be expressed in *Bacillus*, particularly *B. subtilis*, and other gram-positive organisms under the transcriptional control of coliphage T5 or T7-derived promoters and *E. coli*-derived terminators.

In this invention T5 and T7 promoters are defined as promoter function mediating DNA sequences occurring in genomes of the coliphage T5 and T7 family and functional combinations derived from such sequences.

T5 promoters useful in the present invention are those of the "preearly" "early" and "late" expression class of the phage, especially the sequences described in the dissertation of R. Gentz, Universität Heidelberg, 1984: P<sub>J5</sub>, P<sub>N25</sub>, P<sub>N26</sub>, P<sub>D/E20</sub>, P<sub>G5</sub>, P<sub>G20</sub>, P<sub>G22</sub>, P<sub>G25</sub>, P<sub>G28</sub>, P<sub>K28a</sub>, P<sub>K28b</sub>.

The T7 promoters useful in the present invention include the "early" expression class of the phage, especially the promoters A1 and A2 (Hawley, D.K. and McClure, W.D., *Nucleic Acids Res.* 11, 2237-2255 [1983]).

The DNA sequences of some of the preferred T5 or T7 promoters mentioned above are indicated in Table I below:

Table I

	A:T Box	-35	-10	
<sup>s</sup> P <sub>D/E</sub> 20	ACTGCAAAAATAG	TTGACACCC	TAGCCGATAGGCTTTAAGATGTACCCAGTTCGATGA	Phage T5
P <sub>N</sub> 25	TCATAAAAAATTTAT	TTGCTTTCAGGAAAATTTTCTGTATAATAGATTCATAAATTTGA		
<sup>10</sup> P <sub>N</sub> 26	ACTTAAAAATTCAGTTGCTTAATCCTACAATTCCTGATATAATATTCTCATAGTTTGAA			
P <sub>J</sub> 5	ATATAAAACCGTTATTGACACAGGTGGAAATTTAGAATATACTGTTAGTAAACCTAATG			
<sup>15</sup> P <sub>K</sub> 28a	TAGTTAAAATTGTAGTTGCTAAATGCTTAAATACTTGCTATAATATTTATATAAATTGAT			
P <sub>K</sub> 28b	ATTATAAAGTGTTATTGACATTTTCGCCGCTTAGGTATATACTATTATCATTACAGTTGA			
P <sub>G</sub> 25	AAAAATAAAAATTTCTTGATAAAAATTTCCAATACTATTATAATATTGTTATTAAAGAGG			Phage T7
<sup>20</sup> T7A1	TTATCAAAAAGAGTATTGACTTAAAGTCTAACCTATAGGATACTTACAGCCATCGAGAGG			
T7A2	CACGAAAAACAGGTA	TTGACAACATGAAGTAACATGCAGTAAGATACAAATCGCTAGGTA		

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Table I shows the nucleotide sequence of the promoters used in the present invention. The sequence between -50 and +10 is presented, within which the -35 hexamers and upstream A:T-rich regions are boxed, whilst the -10 hexamers are overlined.

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The ribosome binding site-encoding DNA sequence which is necessary for the initiation of translation in a host cell consists of (1) an ATG translation initiation codon for the amino acid methionine, (2) a sequence of 4 to 12 bases which are complementary to bases at the 3'-end of 16s ribosomal RNA and which is known as the Shine Dalgarno (SD) sequence and (3) a sequence of bases between these two known as the linker region.

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The ribosome binding site-encoding DNA sequences used in the present invention and forming part of it may be provided by ribosome binding site-encoding sequences of gram-positive or gram-negative origin capable of functioning in *Bacillus*, particular *B. subtilis*, and other gram-positive organisms, inclusive of several known ones (J.R. McLaughlin et al., *J. Biol. Chem.* 256, 11283-11291 [1981]; C.P. Moran Jr. et al., *Mol. Gen. Genetics* 186, 339-346 [1982]).

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However the preferred ribosome binding site-encoding sequences used in this invention, are portable ribosome binding site-encoding synthetic DNA sequences (SRBS) with the formula indicated in Table II below:

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Table II

5	SRBS I	5' AGCTTTATATAAGGAGGAGTTAAGCATGCAC AATATATTCCTCCTCAATTCGTACGTGTCGA 3'	3'
10	SRBS II	5' AGCTTGGATTATAAAATTTAGGAGGAATTTAAGCATG ACCTAAATTTTAAATCCTCCTTAAATTC 3'	3'
15	RBS I I, 3A+5A	5' AATTCATTAAAGAGGAGAAATTAAGTATGAGGG GTAATTTCTCCTCTTTAATTGATACTCCCTAG 3'	3'
20	RBS I I	5' AATTCATTAAAGAGGAGAAATTAAGTATGAGAG GTAATTTCTCCTCTTTAATTGATACTCTCCTAG 3'	3'
25	RBS I I, 9A	5' AATTCATTAAAGAGGAGAAATTAAGTATGGAAG GTAATTTCTCCTCTTTAATTGATACCTTCCTAG 3'	3'

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These SRBSs have been constructed in a form so that they can function in conjunction with any desired gene encoding prokaryotic or eukaryotic polypeptides in *Bacillus*, particularly *B. subtilis*, and other gram-positive organisms. The ability to so function renders the SRBS "portable".

35 The transcription termination DNA sequence may be provided by terminators of gram-negative bacterial origin capable of functioning in *Bacillus*, particularly *B. subtilis*, and other gram-positive organisms. The preferred gram-negative terminators used in this invention include the *E. coli*-derived terminators  $t_0$  (M. Rosenberg et al., Proc. Natl. Acad. Sci. USA 73, 717-721 [1976]), T1, T2 (J. Brosius et al., J. Mol. Biol. 148, 107-127 [1981]) and T7 (J.J. Dunn and Studier, F.W., Nucleic Acids Res. 8, 2119-2132 [1980]).

40 The transcription initiation DNA sequences, the portable ribosome binding site-encoding sequences and the transcription termination sequences of the present invention can be obtained in accordance with methods well-known in DNA chemistry including total chemical synthesis of the respective DNA sequence, e.g. in a nucleotide synthesizer.

The invention further comprises expression vectors capable of directing expression of a gene encoding pro- and eukaryotic proteins in a *Bacillus*, particularly *B. subtilis* or another gram-positive organism transformed therewith, containing (a) a gram-positive bacterial expression control DNA sequence having in the downstream direction of transcription the following units: at least one transcription initiation DNA sequence of gram-negative bacterial origin combined with a ribosome binding site encoding DNA sequence of gram-positive or gram-negative origin, optionally a foreign gene encoding prokaryotic or eukaryotic polypeptides and a transcription termination DNA sequence, (b) at least one vector origin of replication and 50 (c) at least one antibiotic resistance gene as well as a process for the manufacture of such expression vectors. The transcription initiation DNA sequence may be provided by a gram-negative promoter. The preferred gram-negative promoters used are coliphage T5 or coliphage T7 promoters with the formula as indicated in Table 1. The ribosome binding site-encoding DNA sequence may be provided by a ribosome binding site-encoding DNA sequences of gram-positive or gram-negative bacterial origin capable of functioning in *Bacillus* particularly *B. subtilis* or other gram-positive organisms inclusive of several known ones (J.R. McLaughlin et al., supra; C.F. Moran Jr. et al., Mol. Gen. Genetics 188, 339-348 [1982]). The preferred ribosome binding site-encoding DNA sequences used are portable ribosome binding site-encoding synthetic DNA sequences with the formula indicated in Table II. The transcription termination DNA 55

sequence may be provided by terminators of gram-negative bacterial origin capable of functioning in *Bacillus*, particularly *B. subtilis*, and other gram-positive organisms. The preferred transcription termination DNA sequences used in this invention include the gram-negative *E. coli* terminators  $t_0$  (M. Rosenberg et al., Proc. Natl. Acad. Sci. USA, 73, 717-721 [1976]), T1, T2 (J. Brosius et al., J. Mol. Biol. 148, 107-127 [1981]) and T7 (J.J. Dunn and Studier, F.W., Nucleic Acids Res. 8, 2119-2132 [1980]). The origin of replication may be of gram-negative and/or gram-positive origin and thus the expression vectors can be employed as shuttle vectors (Ehrlich, S.D., Proc. Natl. Acad. Sci. USA 75, 1433-1438 [1978]; Kreft, J. et al., Molec. gen. Genet. 162, 59-67 [1978]; Michel, B. et al., Gene 12, 147-154 [1980]), which can replicate both in *E. coli* and *Bacillus*, especially *B. subtilis*. Preferred expression vectors using ribosome binding site-encoding synthetic DNA sequences ligated to a coliphage T5 promoter and capable of replicating both in *E. coli* and *B. subtilis* (shuttle vectors) are described in the examples 4 and 5 and 7 to 10.

The expression vectors of the present invention can be constructed using techniques of DNA recombination well-known in the art (see laboratory manual "Molecular Cloning" by Maniatis et al., Cold Spring Harbor Laboratory, 1982) comprising the steps of:

- (a) inserting into an existing cloning vector in the downstream direction of transcription at least one transcription initiation DNA sequence of gram-negative bacterial origin and a ribosome binding site-encoding DNA sequence of gram-positive or gram-negative bacterial origin,
- (b) providing in said cloning vector at least one restriction endonuclease site next to said ribosome binding site-encoding DNA sequence;
- (c) inserting at least one foreign gene encoding prokaryotic or eukaryotic polypeptides into said restriction endonuclease site next to said ribosome binding site-encoding DNA sequence, and
- (d) inserting at least one transcription termination DNA sequence in the downstream direction of said foreign gene encoding prokaryotic or eukaryotic polypeptides.

The vector used to assemble the expression vectors of the present invention may be any convenient plasmid, cosmid, or phage capable of transforming and replicating itself in the host microorganisms. Plasmids suitable for cloning in *B. subtilis* and/or *E. coli* are mentioned e.g., in the laboratory manual "Molecular Cloning" by Maniatis et al., supra, and in the dissertation of J. Palva, University of Helsinki, 1983. Preferred vectors of plasmid origin, used to assemble the expression vectors in this invention are pUB 110 (T.J. Gryczan et al., J. Bacteriol. 134, 318-329 [1978]), pDS 5 and pDS 6 (D. Stueber et al., EMBO J. 3, 3143-3148 [1984]).

Plasmids of the p602 and p25 families are specific examples of plasmidic shuttle vectors of the present invention. Their preparation is described more in detail in examples 1 to 5 and 7 to 10. *B. subtilis* strains containing the especially preferred plasmids of the p25 family (*B. subtilis* BR151 transformed with p25RBSI: p25RBSII; p25<sup>\*</sup>RBSII) were deposited at Deutsche Sammlung von Mikroorganismen (DSMZ) in Göttingen on June 20, 1985 the accession nos being DSM 3350, DSM 3351 and DSM 3352 respectively. *B. subtilis* strains containing the especially preferred plasmids of the p602 family (*B. subtilis* BR 151 transformed with p602/18; p602/19; p602/20; p602/21) were deposited at Deutsche Sammlung von Mikroorganismen (DSMZ) in Göttingen on May 14, 1986 the accession nos being DSM 3723, DSM 3724, DSM 3725 and DSM 3726 respectively.

Foreign genes that may be inserted into the expression vectors of this invention may be selected from a large variety of genes (DNA genes or DNA copies of RNA genes) that encode prokaryotic or eukaryotic polypeptides in vivo and in vitro. For example, such genes may encode enzymes, hormones, polypeptides with immuno-modulatory, anti-viral or anti-cancer properties, antibodies, antigens, and other useful polypeptides of prokaryotic or eukaryotic origin. The preferred foreign genes used in this invention are the genes encoding *E. coli* chloramphenicol acetyltransferase (cat) and mouse dihydrofolate reductase (dhfr).

Examples of proteins which can be expressed by using the improved expression control system of the present invention are dihydrofolate reductase, chloramphenicol acetyltransferase, malaria surface antigens, lymphokines like IL-2, interferons alpha, beta and gamma, insulin and insulin precursors, growth hormones, tissue plasminogen activator, human renin or HTLV-III proteins.

Methods for expressing genes encoding prokaryotic or eukaryotic proteins using the expression vectors, especially shuttle vectors, of this invention are well-known (Maniatis et al., supra). They include transforming an appropriate host with an expression vector having the desired DNA sequence operatively inserted into an expression control DNA sequence of the present invention, culturing the host under appropriate conditions of growth and isolating the desired polypeptide from the culture. Those of skill in the art may select from these known methods those that are most effective for a particular gene expression without departing from the scope of this invention.

The selection of a particular host for use in this invention is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen expression vector, toxicity



of the proteins encoded for by the hybrid plasmid, ease of recovery of the desired protein, expression characteristics, biosafety and costs. Within these general guidelines, examples of useful bacterial hosts are gram-negative and gram-positive bacteria, especially strains of *E.coli* and *B.subtilis*. The most preferred host cell of this invention is *B.subtilis* BR 151 (stocked at The Bacillus Genetic Stock Center under BGSC No. 1A40). However, other *B.subtilis* strains such as *B.subtilis* BD 170 (stocked at The Bacillus Genetic Stock Center under BGSC No. 1A 42) and *B.subtilis* JH646 (stocked at The Bacillus Genetic Stock Center under BGSC No. 1S9) can also be used.

The present invention will be better understood on the basis of the following examples when considered in connection with the following figures:

10 Restriction endonucleases have been abbreviated as follows:

E: EcoRI; Sm: SmaI; B: BamHI; S: Sall; P: PstI; H: HindIII;  
Xh: XhoI; X: XbaI; K: KpnI; Pv: PvuII; A: AccI; Sp: SphI;  
Bg: BglII; D: DraI.

In addition, the following abbreviations have been used:

15 kan: Structural gene for kanamycin nucleotidyl transferase;  
cat: Structural gene for chloramphenicol acetyl transferase;  
dhfr: Structural gene for mouse dihydrofolate reductase;  
bla: Structural gene for beta lactamase;  
CAT: Chloramphenicol Acetyl Transferase protein;  
20 DHFR: Dihydrofolate Reductase protein;  
ori +: Gram positive origin of replication;  
ori -: Gram negative origin of replication;  
SRBS: portable ribosome Binding Site-encoding synthetic DNA sequence;  
RBS: Ribosome Binding Site-encoding DNA sequence;  
25 SD: Shine Dalgarno sequence;  
t<sub>0</sub>, T1, T2, T7: Transcriptional terminator to, T1, T2, T7; and  
(H): Cohesive terminus of a HindIII which may be ligated to a HindIII terminus, without generating a HindIII site

30 Figure 1 Construction of the basic *E.coli*/*B.subtilis* shuttle vector p602/5, containing gram-positive (ori +) and gram-negative (ori-) origins of replication, together with drug resistance markers kanamycin (kan) and chloramphenicol. As such, this plasmid confers kanamycin resistance in both *E.coli* and *B.subtilis*. Chloramphenicol resistance is achieved through insertion of promoter-containing fragments between the EcoRI (E) and HindIII (H) sites. The *E.coli* cat gene presented here has its natural ribosome binding site-encoding DNA sequence.

35 Figure 2 Construction of the general expression vectors p602/7 and p602/25, together with the vectors p602/7RBSI and p602/7RBSII containing the ribosome binding site-encoding DNA sequences SRBSI resp. SRBSII. Insertion of the ribosome binding site-encoding DNA sequences SRBSI resp. SRBSII leads to the synthesis of two CAT-type proteins in *E.coli*, i.e. natural CAT protein from the wild type cat ribosomal binding site-encoding DNA sequence and an in-frame fusion CAT protein originating from SRBSI or SRBSII. In *B.subtilis*, a single fusion CAT protein is produced, originating from the ribosome binding site-encoding DNA sequences SRBSI resp. SRBSII. Plasmids p602/7, p602/25, p602/7RBSI and p602/7RBSII all confer chloramphenicol resistance in *E.coli* and *B.subtilis*.

45 Figure 3 Construction of vectors p25RBSI, p25RBSII and p25<sup>\*</sup>RBSII containing the coliphage T5 promoter P<sub>G25</sub> combined with the ribosome binding site-encoding synthetic DNA sequences SRBSI resp. SRBSII. *B.subtilis* cells containing the vector p25RBSI synthesise a single CAT fusion protein, originating in the immediate downstream vicinity of SRBSI. *B.subtilis* cells containing the vector p25RBSII synthesise two fusion CAT proteins, originating at the immediate downstream vicinity of SRBSII, as well as a longer fusion protein originating from a ribosome binding site in the immediate vicinity of P<sub>G25</sub>. Protein synthesis originating from this additional ribosome binding site was eliminated by providing a translational termination codon upstream from SRBSII, resulting in the vector p25<sup>\*</sup>RBSII. Cells containing p25<sup>\*</sup>RBSII now synthesise a single fusion CAT protein, originating from SRBSII.

55 Figure 4 Total proteins synthesized in *B.subtilis* strain BR151 containing the expression vectors p25RBSI, p25RBSII and p25<sup>\*</sup>RBSII. The position of the CAT protein originating from SRBSI or SRBSII is indicated 'CAT'; the additional fusion CAT protein from cells harbouring p25RBSII is indicated 'f-CAT'. LYS indicates lysozyme, which is added externally to aid cell lysis.

**Figur 5** Diagrammatic representation of CAT proteins synthesised in *B.subtilis* containing the vectors p25RBSI, p25RSSII and p25<sup>\*</sup>RBSII. An in-frame translational stop codon (  $\lambda$  ) prevents readthrough protein synthesis into the cat gene from P<sub>G25</sub> RBS. Such an in-frame stop codon is absent in the construction p25RBSII; consequently, cat proteins arise from RBS and SRBSII. Modification of the HindIII site in p25<sup>\*</sup>RBSII introduces an in-frame stop codon, and, as a consequence, yields a single CAT protein from SRBSII.

**Figure 6** In vitro transcriptional analysis of the promoters presented in Table 1. The notations 'Ec' and 'Bs' indicate analysis with *E. coli* and *B.subtilis* RNA polymerase, respectively, and the figures in conjunction the salt concentration at which the transcription was performed. 'ori' and 'bla' transcripts arise from the vector into which the promoters were cloned. The panel indicated 'veg' represents transcription of solely the *B.subtilis* veg promoter (Le Grice, S.F.J. and Sonenshein, A.L. J.Mol.Biol., 162, 551-564, 1982). 'veg' indicated at the side of the panel indicates transcription of internally supplied veg promoter DNA. M, molecular weight marker, HpaII-cleaved pBR322 DNA. Only the sizes of bands relevant to the present research have been presented. Note that the panel illustrating transcription from the T5 promoter P<sub>K28a</sub>/P<sub>K28b</sub> has two new transcripts, as both promoters are present on a single restriction fragment.

**Figure 7** Construction of the shuttle vectors p602/18 and p602/19, containing the coliphage T5 promoter P<sub>N25</sub> operably linked to either the synthetic ribosome binding site-encoding DNA sequence RBSII, 9A (p602/18) or RBSII, 3A + 5A (p602/19). Insertion of the synthetic ribosome binding site-DNA encoding sequences leads, in both cases, to synthesis of a fusion CAT protein initiating in the immediate vicinity of the synthetic ribosome binding site and terminating at the natural translational stop codon of the cat gene. Plasmids p602/18 and 602/19 both confer chloramphenicol resistance on *B.subtilis*.

**Figure 8** Construction of the shuttle vectors p602/20 and p602/21, containing the coliphage T5 promoter P<sub>N25</sub> operably linked to the synthetic ribosome binding site-encoding DNA sequences RBSII (p602/20) or RBSII, 3A + 5A (p602/21). Insertion of the synthetic ribosome binding Site-DNA encoding sequences leads, in both cases to synthesis of a fusion DHFR protein, initiating in the immediate vicinity of the synthetic ribosome binding site and terminating at the natural translational termination codon of the dhfr gene. *B.subtilis* cells containing 602/20 or 602/21 are resistant to 10 µg/ml trimethoprim.

**Figure 9** Total proteins synthesised in *B.subtilis* strain BR151 containing the plasmids p602/18, p602/19, p602/20 and p602/21. Cell denotes protein synthesis from plasmid-free cells. As reference, CAT synthesis from p25<sup>\*</sup>RBSII has been included. The positions of the fusion CAT protein CAT<sup>\*</sup> (from p602/18 and p602/19) and fusion DHFR protein (from p602/20 and p602/21) have been indicated.

#### General Methods

The following methods were performed as described by Maniatis et al., supra, unless indicated differently: Restriction endonuclease digestions at 37 °C (pp. 100-101); dephosphorylation with bacterial alkaline phosphatase (BAP) at 37 °C (pp. 133-134); ligation with T4 DNA ligase at 14 °C (pp. 390-391); transformation of DNA into CaCl<sub>2</sub>-cells of *E.coli* HB101 and selection of transformants on agar plates containing LB-medium plus 100 µg/ml of ampicillin (pp. 250-251); DNA plasmid preparation (pp. 86-94); filling-in single-stranded DNA-tails with the large fragment of DNA polymerase I (Klenow fragment) at 14 °C (pp. 113-114); DNA separation and fragment purification from agarose gels (pp. 164-167); the use of synthetic DNA linkers in subcloning (pp. 392-397); and SDS/Polyacrylamide gel electrophoresis (pp.348-349).

Transformation of DNA into cells of *B. subtilis* was performed as described by S. Contente and Dobnau, D. (Mol. Gen. Genet 167, 251-258 [1979]).

In vitro transcription with RNA polymerases of *E. coli* and *B.subtilis* was performed in 50 µl assays of the following composition; 40 mM Tris/HCl, pH 7.9, 10 mM MgCl<sub>2</sub>, 0.1 mM DTT, 0.1 mM EDTA, 50-200 mM NaCl, 10% (v/v) glycerol, 150 µM ATP, GTP, CTP, 50 µM UTP, 5 µCi <sup>32</sup>P-UTP ( ~ 3000 Ci/m mole, Amersham Buchler, Braunschweig), 0.05 p mole endonucleolytically-cleaved DNA, 0.25 p mole RNA polymerase. Reactions were initiated by addition of RNA polymerase and allowed to proceed for between 1 and 5 mins at 37 °C. Synthesised RNA was isolated by repeated ethanol precipitation and analysed by high voltage gel electrophoresis through 0.4 mm thick 5 or 8% polyacrylamide gels containing 8M urea.

Following electrophoresis, gels were dried and subjected to autoradiography using Kodax X-OMAT XAR 5 film at room temperature.

## 5 Example 1

### Construction of Shuttle vector P602/5

- 10 (I) 2  $\mu$ g of plasmid pUB110 are digested to completion with the restriction endonuclease Pvu II. An octameric KpnI linker is ligated to the Pvu II ends. Following ligation, the DNA is digested to completion with the endonucleases KpnI and EcoRI. The resulting digested DNA is electrophoresed through a 1% low melting temperature agarose gel containing 1  $\mu$ g/ml ethidium bromide. After 2 hours electrophoresis at 70V, the DNA bands are visualized by fluorescence, and the upper 3.5 Kb band is excised from the gel. This 3.5 Kb, EcoRI/KpnI fragment is subsequently purified from the low melting temperature agarose.
- 15 (II) 5  $\mu$ g of plasmid pDS5 are cleaved to completion with DraI, and radioactive octameric KpnI linkers are ligated to the DraI termini. The products of ligation are subsequently cleaved to completion with the endonucleases KpnI and XbaI and separated by electrophoresis through a 6% polyacrylamide gel. A KpnI/XbaI fragment of approximately 1.2 Kb is located by autoradiography and excised from the gel. The KpnI/XbaI fragment is subsequently purified from the acrylamide gel slice.
- 20 (III) 5  $\mu$ g of plasmid pDS5 are digested to completion with the endonucleases EcoRI and XbaI, then separated by electrophoresis through a 1% low melting temperature agarose gel containing 1  $\mu$ g/ml ethidium bromide. Following electrophoresis, the DNA bands are visualized by fluorescence, and an approximately 900 bp EcoRI/XbaI fragment is excised. The EcoRI/XbaI fragment is subsequently purified from the low melting temperature agarose.
- 25 (IV) Equimolar amounts of the purified DNA fragments from steps (I) to (III) are ligated, and the products of the ligation transformed into competent cultures of E.coli strain AB1157 (Maniatis et al., supra). Transformed cells are plated on LB agar containing 10  $\mu$ g/ml kanamycin. Plasmid DNA is isolated from kanamycin-resistant colonies and the integrity of the respective fragments verified by restriction endonuclease cleavage. Plasmid thus generated is designated p602/5. The construction of p602/5 is illustrated in Figure 1.
- 30

## 35 Example 2

### Construction of the expression vector p602/7RBSI and p602/7RBSII carrying portable ribosome binding site-encoding synthetic DNA sequences.

- 40 (I) 2  $\mu$ g of plasmid p602/5 are digested to completion with the restriction endonucleases EcoRI and HindIII and the approximately 5.6Kb vector DNA fragment is isolated. This fragment is thereafter ligated with a 125 bp EcoRI/HindIII fragment containing the B.subtilis promoter P<sub>VII</sub>, having the following DNA sequence:

45 5' AATTCTCATG TTTGACAGCT TATCATCGAA TTATAGGAAT AGAGCAAACA  
3' GAGTAC AAACGTGTCGA ATAGTAGCTT AATATCCTTA TCTCGTTTGT

50 AGCAAAGGAA ATTTTGTCAA AATAATTTTA TTGACAACGT CTTATTAACG  
TCGTTTCCTT TAAACAGTT TTATTAAAAA AACTGTTGCA GAATAATTGC

55 TTGATATAAT TTGCA 3'  
AACTATATTA AACGTTTCGA 5'

The ligation products are transformed into E.coli strain AB1157 and transformed cells are selected on LB agar containing 50 µg/ml chloramphenicol. Chloramphenicol-resistant colonies are analysed to verify the insertion of promoter P<sub>VI</sub> into the plasmid p602/5. The resultant plasmid is designated p602/7. (II) 2 µg of plasmid p602/7 are digested to completion with the endonuclease HindIII. The portable ribosome binding site-encoding synthetic DNA sequence SRBSI, having the sequence

```

5'                                     3'
AGCTTTATATAAGGAGGAGTTAAGCATGCAC
AATATATTCCTCCTCAATTCGTACGTGTCGA
3'                                     5'

```

is ligated into the HindIII site. The ligation products are transformed into E.coli strain AB1157 and transformed cells are selected on LB agar containing 50 µg/ml chloramphenicol. Chloramphenicol-resistant colonies are assayed for acquisition of plasmids containing the portable ribosome binding site-encoding synthetic DNA sequence SRBSI via

a) ability to synthesise a fusion CAT protein from SRBSI

b) restriction enzyme analysis of the recombinant plasmid, which harbours a newly acquired SphI site.

Plasmid DNA thus characterised is designated p602/7RBSI. Purified p602/7RBSI DNA is thereafter transformed into B.subtilis strain BR151 and chloramphenicol-resistant colonies (in this case, colonies resistant to 10 µg/ml chloramphenicol) are assayed as mentioned in (II) a) and b) to verify the utility of SRBSI in B.subtilis.

(III) Plasmid p602/7RBSI is digested to completion with HindIII and SphI and purified from SRBSI by electrophoresis through a 1% low melting temperature agarose gel containing 1 µg/ml ethidium bromide. Following electrophoresis, the DNA is visualized by fluorescence and excised from the gel. DNA is subsequently purified from the agarose. A portable ribosome binding site-encoding synthetic DNA sequence, designated SRBSII, and having the sequence

```

5'                                     3'
AGCTTGGATTAAATTTAGGAGGAATTTAAGCATG
ACCTAAATTTAAATCCTCCTTAAATTC
3'                                     5'

```

is ligated with HindIII/SphI cleaved p602/7RBSI DNA. E.coli strain AB1157 is transformed with this ligation mixture, and transformed cells are selected on LB agar containing 50 µg/ml chloramphenicol. Chloramphenicol-resistant colonies are assayed for the presence of SRBSII by

a) ability to synthesise a fusion CAT protein

b) restriction enzyme analysis of the recombinant plasmid, which harbours a new DraI site.

Plasmid DNA thus characterised is designated p602/7RBSII. Plasmid p602/7RBSII is introduced into competent cells of B.subtilis strain BR151 and transformed cells are selected on LB agar containing 10 µg/ml chloramphenicol. Chloramphenicol-resistant colonies are analysed for the utility of SRBSII in B.subtilis as described in Step (III) a) and b). The construction of vectors p602/7RBSI and p602/7RBSII is illustrated in Figure 2.

### Example 3

#### Construction of Expression Vector p602/25 carrying the coliphage T5 promoter P<sub>G25</sub>.

(I) 2µg of plasmid p602/5 are digested to completion with the restriction endonuclease EcoRI. This DNA is thereafter ligated with equimolar amounts of a 250bp EcoRI fragment containing the coliphage T5 promoter P<sub>G25</sub> (R. Gentz, supra). The ligated products are transformed into E.coli strain AB1157, and transformed cells are selected on LB agar containing 100 µg/ml chloramphenicol. Plasmid DNA is isolated from chloramphenicol-resistant colonies and analysed by EcoRI digestion, or DNA sequencing, for the presence of the 250bp fragment containing promoter P<sub>G25</sub>. Plasmid DNA thus characterised is

designated p602/25. The construction of p602/25 is illustrated in Figure 2.

#### Example 4

Construction of the Vector p25RBSI carrying the coliphage T5 promoter P<sub>G25</sub> combined with the portable ribosome binding site-encoding synthetic DNA sequence SRBSI.

(I) 2 µg of plasmid p602/7RBSI are digested to completion with the restriction endonucleases HindIII and BglII, and fractionated by electrophoresis through a 1% low melting temperature agarose gel containing 1 µg/ml ethidium bromide. Following electrophoresis, the DNA bands are visualized by fluorescence, and the upper, approximately 3.2Kb band is excised. This fragment is then purified from the agarose.

(II) 2 µg of plasmid p602/25 are likewise digested to completion with the restriction endonucleases HindIII and BglII, and fractionated by electrophoresis through a 1% agarose gel containing ethidium bromide. The DNA bands are visualised by fluorescence and the lower, approximately 2.6Kb band is excised and purified from the agarose.

(III) Equimolar amounts of the DNA fragments prepared through Example 4, (I) and (II) are ligated, and the ligation products are transformed into E.coli strain AB1157. Plasmid DNA is isolated from colonies resistant to 100 µg/ml chloramphenicol and analysed for the presence of the 250bp EcoRI band. Plasmid DNA thus characterised is designated p25RBSI. The construction of plasmid p25RBSI is illustrated in Figure 3.

(IV) plasmid DNA is isolated from E.coli harbouring p25RBSI and transformed into competent cultures of B.subtilis strain BR151 and transformed cells are selected on LB agar containing 10 µg/ml chloramphenicol. Plasmid DNA is isolated from chloramphenicol resistant colonies and the structure of plasmid p25RBSI in B.subtilis verified by restriction endonuclease analysis.

(V) B.subtilis colonies containing plasmid p25RBSI are cultivated in L-Broth containing 10 µg/ml chloramphenicol, and total protein synthesized by these cultures is analysed by SDS/polyacrylamide gel electrophoresis. Utilisation of the coliphage T5 promoter P<sub>G25</sub> together with the synthetic ribosome binding site-encoding DNA sequence SRBSI is verified by synthesis of a fusion CAT protein, initiating in the immediate vicinity of SRBSI and terminating at the natural translational termination codon of the E.coli cat gene. The results of such an analysis are presented in Figure 4.

#### Example 5

Construction of the Vectors p25RBSII and p25RBSII carrying the coliphage T5 promoter P<sub>G25</sub> combined with the portable ribosome binding site-encoding synthetic DNA sequence SRBSII.

(I) 2 µg of plasmid p602/7RBSII are digested to completion with the restriction endonucleases HindIII and BglII, and the products fractionated by electrophoresis through a 1% low melting temperature agarose gel containing 1 µg/ml ethidium bromide. Following electrophoresis, the DNA bands are visualized by fluorescence; the upper, approximately 3.2Kb band is excised and purified from the agarose.

(II) 2 µg of the plasmid p602/25 are similarly digested to completion with the restriction endonucleases HindIII and BglII and fractionated by electrophoresis through a 1% low melting temperature agarose gel containing 1% ethidium bromide. Following electrophoresis, the DNA bands are visualized by fluorescence and the lower, approximately 2.6Kb band is excised and purified from the gel. Equimolar amounts of the DNA fragments isolated through Example 5 (I) and (II) are ligated, and the ligation products transformed into E.coli strain AB1157; transformed cells are selected on LB agar containing 100 µg/ml chloramphenicol. Plasmid DNA is isolated from chloramphenicol resistant colonies, and the presence of both the coliphage T5 promoter P<sub>G25</sub> as well as the synthetic ribosome binding site-encoding DNA sequence SRBSII are verified by restriction endonuclease analysis. Plasmid DNA thus characterised is designated p25RBSII. The construction of plasmid p25RBSII is illustrated in Figure 3.

Protein synthesis in B.subtilis containing the vector p25RBSII is illustrated in Figure 4. It was discovered here that the EcoRI fragment harbouring the promoter P<sub>G25</sub> contains an accessory ribosome binding site, which produces a fusion protein extending to the end of the cat gene. The immediate effect is to drastically reduce the efficiency of RBSII; as a consequence, the protein reading frame from the ribosome binding site in the immediate vicinity of P<sub>G25</sub> was altered as follows, to maximise protein synthesis from SRBSII:

(IV) 2 µg of plasmid p25/RBSII are digested to completion with the restriction endonuclease HindIII. The cohesive HindIII termini are converted to blunt termini by incubation with DNA polymerase Klenow fragment in the presence of all four dNTPs. Duodecameric HindIII linkers are ligated to these blunt termini, and the ligation products digested to completion with HindIII. This DNA is fractionated by electrophoresis through a 1% low melting temperature agarose gel containing 1 µg/ml ethidium bromide. Following electrophoresis, the DNA is visualized by fluorescence, excised from the gel and purified from the agarose. This DNA is again ligated, and the ligation products transformed into E.coli strain AB1157. Transformed cells are selected on LB agar containing 100 µg/ml chloramphenicol. Plasmid DNA is isolated from chloramphenicol resistant colonies, and the presence of the newly introduced HindIII site verified by restriction endonuclease analysis. Plasmid DNA thus characterised is designated p25<sup>+</sup>RBSII. The construction of plasmid p25<sup>+</sup>RBSII is illustrated in Figure 3.

(V) Plasmid p25<sup>+</sup>RBSII is introduced into competent cultures of B.subtilis strain BR151, and transformed cells are selected on LB agar containing 10 µg/ml chloramphenicol. Plasmid DNA is then isolated from chloramphenicol resistant colonies and its structural identity to p25<sup>+</sup>RBSII isolated from E.coli is determined by restriction endonuclease analysis.

(VI) Individual chloramphenicol resistant colonies of B.subtilis are cultivated in L-Broth containing 10 µg/ml chloramphenicol, and total protein synthesized by these colonies is analysed by SDS/polyacrylamide gel electrophoresis. The utilisation of the coliphage T5 promoter P<sub>G25</sub>, together with the synthetic ribosome binding site-encoding DNA sequence SRBSII, is verified by the synthesis of a fusion CAT protein, initiating in the immediate vicinity of SRBSII and terminating at the natural termination codon of the E.coli cat gene. The results of such an analysis are presented in Figure 5.

#### Example 6

In vitro analysis of E.coli promoters with B.subtilis RNA polymerase.

Table 1 indicates the promoters which were used. Their potential was determined by in vitro 'run-off' transcription, the results of which are presented in Figure 6. In each case, promoter utilisation by B.subtilis  $\sigma^{55}$  RNA polymerase has been determined as a function of increasing ionic strength, and compared with its efficiency when transcribed with E.coli RNA polymerase at 200 mM NaCl. Each transcription assay contains, in addition to the promoter in question, stoichiometric amounts of the B.subtilis veg promoter, previously shown to be efficiently utilised by B.subtilis  $\sigma^{55}$  RNA polymerase (Moran Jr. et al., Mol. Gen. Genetics 186, 339-346 [1982]). It is clear from the data of Figure 6 that all promoters tested are recognised by B.subtilis RNA polymerase, albeit to varying degrees. In the case of the coliphage T5 promoters P<sub>N28</sub> and P<sub>K28a</sub>/P<sub>K28b</sub>, transcription may in fact be stronger than that from the veg promoter. Furthermore, the effect of salt concentration on promoter efficiency is clear. At 50 mM NaCl, B.subtilis RNA polymerase initiates transcription not only from the promoters in question, but also from the 'bla' and 'ori' promoters of the pBR322 vector DNA (for preliminary studies, all promoters were inserted into pBR322 derived vectors: these plasmids were subsequently cleaved to yield a constant 350 nucleotide 'bla' transcript and a variable length transcript from the coliphage T5 promoter in question). As the salt concentration is raised, promoter selection becomes clearly evident partitioning between the veg and coliphage T5 promoters. To test whether the results of Figure 6 have in vivo relevance, coliphage T5 promoters, or the A1 promoter of coliphage T7, can be substituted for the P<sub>G25</sub> promoter of the vector p25<sup>+</sup>RBSII (Figure 3), and CAT synthesis in B.subtilis can be determined.

#### Example 7

Construction of the Vector D602/18 carrying the coliphage T5 promoter P<sub>N25</sub> combined with the portable ribosome binding site-encoded synthetic DNA sequence RBSII, 9A.

(I) 2 µg of the plasmid pDS5/RBSII, 9A are digested to completion with the restriction endonucleases XhoI and XbaI, and fractionated by electrophoresis through a 1% low melting temperature agarose gel containing 1 µg/ml ethidium bromide. Following electrophoresis, the DNA bands are visualised by fluorescence and the lower, approximately 1.0kb band is excised. This fragment is then purified from the agarose.

(II) 2 µg of the plasmid p25<sup>+</sup>RBSII are likewise digested to completion with the restriction endonucleases XhoI and XbaI, and fractionated through a 1% low melting temperature agarose gel containing 1 µg/ml ethidium bromide. The bands are visualised by fluorescence and the upper, approximately 4.6Kb band excised and purified from the agarose.

5 (III) Equimolar amounts of the DNA fragments prepared through Example 7, (I) and (II) are ligated, and the ligation products are transformed into competent cells of *B. subtilis* strain BR151. Plasmid DNA is isolated from transformed cells resistant to 10 µg/ml kanamycin and 10 µg/ml chloramphenicol and analyzed for the presence of the 1.0kb XhoI/XbaI fragment. Plasmid thus characterised is designated p602/18. The construction of p602/18 is illustrated in Figure 7.

10 (IV) *B. subtilis* colonies containing plasmid p602/18 are cultivated in L-Broth containing 10 µg/ml chloramphenicol, and total protein synthesised by these cultures is analysed by SDS/polyacrylamide gel electrophoresis. Utilisation of the coliphage promoter P<sub>N25</sub> together with the synthetic ribosome binding site-encoding DNA sequence RBSII, 9A, is verified by the synthesis of a fusion CAT protein, initiating in the immediate vicinity of RBSII, 9A and terminating at the natural translational termination codon of the

15 *E. coli* cat gene. The results of such an analysis are presented in Figure 9.

#### Example 8

20 Construction of the vector p602/19, carrying the coliphage T5 promoter P<sub>N25</sub> combined with the portable ribosome binding site encoding synthetic DNA sequence RBSII, 3A + 5A.

(I) 2 µg of the plasmid pDS5/RBSII, 3A+5A are digested to completion with the restriction endonucleases XhoI and XbaI and fractionated by electrophoresis through a 1% low melting temperature agarose gel containing 1 µg/ml ethidium bromide. Following electrophoresis, the DNA bands are visualized by fluorescence, and the lower, approximately 1.0Kb band is excised. This fragment is then purified from the agarose.

25

(II) 2 µg of plasmid p25<sup>+</sup>RBSII are likewise digested to completion with the restriction endonucleases XhoI and XbaI and fractionated by electrophoresis through a 1% low melting temperature agarose gel containing 1 µg/ml ethidium bromide. The DNA bands are visualised by fluorescence and the upper, approximately 4.7Kb band is excised and purified from the agarose.

30

(III) Equimolar amounts of the DNA fragments purified through Example 8, (I) and (II) are ligated, and the ligation products are transformed into competent cells of the *B. subtilis* strain BR151. Plasmid DNA is purified from transformants resistant to 10 µg/ml kanamycin and 10 µg/ml chloramphenicol and assayed for the presence of the 1.0Kb XhoI/XbaI fragment. Plasmid DNA thus characterised is designated p602/19. The construction of p602/19 is illustrated in Figure 7.

35

(IV) *B. subtilis* colonies containing plasmid p602/19 are cultivated in L-Broth containing 10 µg/ml chloramphenicol, and total protein synthesised by these cultures is analysed by SDS/polyacrylamide gel electrophoresis. Utilisation of the coliphage T5 promoter P<sub>N25</sub> together with the synthetic ribosome binding site-encoding DNA sequence RBSII, 3A + 5A is verified by synthesis of a fusion CAT protein, initiating in the immediate vicinity of RBSII, 3A + 5A, and terminating at the natural translation termination codon of the cat gene. The results of such an analysis are presented in Figure 9.

40

#### Example 9

45

Construction of the vector p602/20 carrying the coliphage T5 promoter P<sub>N25</sub> combined with the portable ribosome binding site-encoding DNA sequence RBSII.

50 (I) 2 µg of the plasmid pDS8/RBSII are digested to completion with the restriction endonucleases XhoI and XbaI, and fractionated by electrophoresis through a 1% low melting temperature agarose gel containing 1 µg/ml ethidium bromide. Following electrophoresis, the DNA bands are visualised by fluorescence, and the lower, approximately 2.0Kb band is excised. The fragment is then purified from the agarose.

55 (II) 2 µg of the plasmid p25<sup>+</sup>RBSII are likewise digested to completion with the restriction endonucleases XhoI and XbaI and fractionated by electrophoresis through a 1% low melting temperature agarose gel containing 1 µg/ml ethidium bromide. The DNA bands are visualised by fluorescence, and the upper, approximately 4.7Kb band is excised and purified from the agarose.

(III) Equimolar amounts of the DNA fragments prepared through Example 9 (I) and (II) are ligated, and the ligation products are transformed into competent cells of the *B.subtilis* strain BR151. Plasmid DNA is purified from transformants resistant to 10 µg/ml kanamycin and 10 µg/ml trimethoprim and analysed for the presence of the 2.0Kb XhoI/XbaI fragment. Plasmid DNA thus characterised is designated p602/20.

The construction of p602/20 is illustrated in Figure 8.

(IV) *B.subtilis* colonies containing plasmid p602/20 are cultivated in L-Broth containing 10 µg/ml kanamycin, and total protein synthesized by these cultures is analysed by SDS/polyacrylamide gel electrophoresis. Utilisation of the coliphage T5 promoter  $P_{N25}$  together with the synthetic ribosome binding site-encoding DNA sequence RBSII is verified by the synthesis of a fusion DHFR protein, initiating in the immediate vicinity of RBSII and terminating at the natural translational termination codon of the dhfr gene. The results of this analysis are presented in Figure 9.

#### Example 10

Construction of the vector p602/21 containing the the coliphage T5 promoter  $P_{N25}$  combined with the portable ribosome binding site-encoding DNA sequence RBSII, 3A + 5A.

(I) 2 µg of the plasmid pDS8/RBSII,3A + 5A are digested to completion with the restriction endonucleases XhoI and XbaI, and the products fractionated through a 1% low melting temperature agarose gel containing 1 µg/ml ethidium bromide. Following electrophoresis, the DNA bands are visualised by fluorescence, and the lower, approximately 2.0Kb band excised and purified from the agarose.

(II) 2 µg of the plasmid p25<sup>+</sup>RBSII are likewise digested to completion with the restriction endonucleases XhoI and XbaI, and the products fractionated through a 1% low melting temperature agarose gel containing 1 µg/ml ethidium bromide. Following electrophoresis, the DNA bands are visualized by fluorescence, and the lower, approximately 2.0Kb band excised and purified from the agarose.

(III) Equimolar amounts of the DNA fragments purified through Example 10 (I) and (II) are ligated, and the ligation products transformed into competent cells of the *B.subtilis* strain BR151. Plasmid DNA is isolated from transformants resistant to 10 µg/ml kanamycin and 10 µg/ml trimethoprim and assayed for the presence of the 2.0Kb XhoI/XbaI fragment. Plasmid DNA thus characterised is designated p602/21. The construction of p602/21 is illustrated in Figure 8.

(IV) *B.subtilis* colonies containing plasmid p602/21 are cultivated in L-Broth containing 10 µg/ml kanamycin, and total protein synthesized by these cultures is analysed by SDS/polyacrylamide gel electrophoresis. Utilisation of the coliphage T5 promoter  $P_{N25}$  together with the synthetic ribosome binding site-encoding DNA sequence RBSII, 3A + 5A is verified by the synthesis of a fusion DHFR protein, initiating in the immediate vicinity of RBSII, 3A + 5A and terminating at the natural termination codon of the dhfr gene. The results of such an analysis are presented in Figure 9.

#### Claims

1. A gram-positive bacterial expression control DNA sequence comprising in the downstream direction of transcription:

- (a) a coliphage T5 or T7 promoter;
- (b) a ribosome binding site-encoding synthetic DNA sequence with the formula



5' 3'  
 AGCTTTATATAAGGAGGAGTTAAGCATGCAC (SRBSI),  
 5 AATATATTCCTCCTCAATTCGTACGTGTCGA  
 3' 5'  
 5' 3'  
 10 AGCTTGGATTTAAAATTTAGGAGGAATTTAAGCATG (SRBSII),  
 ACCTAAATTTTAAATCCTCCTTAAATTC  
 3' 3'  
 15 5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGAGGG (RBSII, 3A+5A),  
 20 GTAATTTCTCCTCTTTAATTGATACTCCCCTAG  
 3' 5'  
 25 5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGAGAG (RBSII) or  
 GTAATTTCTCCTCTTTAATTGATACTCTCCTAG  
 3' 5'  
 30 5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGGAAG (RBSII, 9A);  
 35 GTAATTTCTCCTCTTTAATTGATACCTTCCTAG  
 3' 5'

and  
 40 (c) a transcription termination DNA sequence of gram-negative origin.

2. An expression control DNA sequence according to claim 1, wherein said coliphage T5 promoter is the P<sub>N25</sub> promoter.
- 45 3. An expression control DNA sequence according to claim 1, wherein said coliphage T5 promoter is the P<sub>N28</sub> promoter.
4. An expression control DNA sequence according to claim 1, wherein said coliphage T5 promoter is the P<sub>Q25</sub> promoter.
- 50 5. An expression control DNA sequence according to claim 1, wherein said coliphage T5 promoter is the P<sub>J5</sub> promoter.
6. An expression control DNA sequence according to claim 1, wherein said coliphage T5 promoter is the P<sub>D/E20</sub> promoter.
- 55 7. An expression control DNA sequence according to claim 1, wherein said coliphage T5 promoter is the P<sub>K28a</sub> promoter.

8. An expression control DNA sequence according to claim 1, wherein said coliphage T5 promoter is the  $P_{K285}$  promoter.
9. An expression control DNA sequence according to claim 1, wherein said T7 promoter is the T7A1 promoter.
10. An expression control DNA sequence according to claim 1, wherein said T7 promoter is the T7A2 promoter.
11. An expression control DNA sequence according to any one of claims 1 to 10, wherein said transcription termination DNA sequence is the E. coli  $t_0$  terminator.
12. An expression control DNA sequence according to any one of claims 1 to 10, wherein said transcription termination DNA sequence is the E. coli T1 terminator.
13. An expression control DNA sequence according to any one of claims 1 to 10, wherein said transcription termination DNA sequence is the E. coli T2 terminator.
14. An expression control DNA sequence according to any one of claims 1 to 10, wherein said transcription termination DNA sequence is the E. coli T7 terminator.
15. An expression control DNA sequence according to any one of claims 1 to 14, capable of functioning in Bacillus.
16. An expression control DNA sequence according to claim 15, capable of functioning in Bacillus subtilis.
17. A ribosome binding site-encoding synthetic DNA sequence, capable of functioning in gram-positive organisms with the formula

5' 3'  
 AGCTTTATATAAGGAGGAGTTAAGCATGCAC (SRBSI),  
 5 AATATATTCCTCCTCAATTCGTACGTGTCGA  
 3' 5'

5' 3'  
 10 AGCTTGGATTTAAAATTTAGGAGGAATTTAAGCATG (SRBSII),  
 ACCTAAATTTTAAATCCTCCTTAAATTC  
 3' 3'

15 5' 3'  
 AATTCATTAAAGAGGAGAGAAATTAACATGAGGG (RBSII, 3A+5A),  
 20 GTAATTTCTCCTCTTTAATTGATACTCCCCTAG  
 3' 5'

25 5' 3'  
 AATTCATTAAAGAGGAGAGAAATTAACATGAGAG (RBSII) or  
 GTAATTTCTCCTCTTTAATTGATACTCTCCTAG  
 3' 5'

30 5' 3'  
 AATTCATTAAAGAGGAGAGAAATTAACATGGAAG (RBSII, 9A);  
 35 GTAATTTCTCCTCTTTAATTGATACCTTCCTAG  
 3' 5'

- 40 18. A ribosome binding site-encoding synthetic DNA sequence according to claim 17, capable of functioning in *Bacillus*.
19. A ribosome binding site-encoding synthetic DNA sequence according to claim 18, capable of functioning in *Bacillus subtilis*.
- 45 20. An expression vector containing  
 (a) a gram-positive bacterial expression control DNA sequence as claimed in any one of claims 1 to 18, wherein said ribosome binding site-encoding DNA sequence is optionally operatively linked to a foreign gene encoding prokaryotic or eukaryotic polypeptides,  
 50 (b) at least one vector origin of replication and  
 (c) at least one antibiotic resistance gene.
21. An expression vector according to claim 20 which is a plasmidic shuttle vector capable of replication in gram-negative and gram-positive bacteria.
- 55 22. An expression vector according to claim 21 which is capable of replication in a strain of *E. coli* and *Bacillus*.

23. An expression vector according to claim 22 which is capable of replication in a strain of *E. coli* and *B. subtilis*.
- 5 24. An expression vector according to any one of claims 20 to 23 which is p25RBSI DSM 3350.
25. An expression vector according to any one of claims 20 to 23 which is p25RBSII DSM 3351.
26. An expression vector according to any one of claims 20 to 23 which is p25RBSII DSM 3352.
- 10 27. An expression vector according to any one of claims 20 to 23 which is p602/18 DSM 3723.
28. An expression vector according to any one of claims 20 to 23 which is p602/19 DSM 3724.
29. An expression vector according to any one of claims 20 to 23 which is p602/20 DSM 3725.
- 15 30. An expression vector according to any one of claims 20 to 23 which is p602/21 DSM 3726.
31. A transformant carrying an expression vector as claimed in any one of claims 20 to 30.
- 20 32. A transformant according to claim 31 which is a *Bacillus subtilis* strain.
33. A transformant according to claim 32 which is a *Bacillus subtilis* BR 151 strain.
34. A process for the manufacture of a gram-positive bacterial expression control DNA sequence as  
25 claimed in any one of claims 1 to 18, which process comprises combining in the downstream direction  
of transcription:  
    (a) a coliphage T5 or T7 promoter;  
    (b) a ribosome binding site-encoding synthetic DNA sequence with the formula

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5' 3'  
 AGCTTTATATAAGGAGGAGTTAAGCATGCAC (SRBSI),  
 AATATATTCCTCCTCAATTCGTACGTGTCGA  
 5' 3'  
 5' 3'  
 AGCTTGGATTTAAAATTTAGGAGGAATTTAAGCATG (SRBSII),  
 ACCTAAATTTTAAATCCTCCTTAAATTC  
 3' 3'  
 5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGAGGG (RBSII, 3A+5A),  
 GTAATTTCTCCTCTTTAATTGATACTCCCCTAG  
 3' 5'  
 5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGAGAG (RBSII) or  
 GTAATTTCTCCTCTTTAATTGATACTCTCCTAG  
 3' 5'  
 5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGGAAG (RBSII, 9A);  
 GTAATTTCTCCTCTTTAATTGATACCTTCCTAG  
 3' 5'

and  
 (c) a transcription termination DNA sequence of gram-negative origin to a functional unit by techniques of DNA recombination well-known in the art.

35. A process for the manufacture of an expression vector as claimed in any one of claims 20 to 30 by techniques of DNA recombination well-known in the art comprising the steps of:
- (a) Inserting into an existing cloning vector in the downstream direction of transcription at least one coliphage T5 or T7 promoter and a ribosome binding site-encoding DNA sequence as defined in claim 17,
  - (b) providing in said cloning vector at least one restriction endonuclease site next to said ribosome binding site-encoding DNA sequence,
  - (c) Inserting at least one foreign gene encoding prokaryotic or eukaryotic polypeptides into said restriction endonuclease site next to said ribosome binding site-encoding DNA sequence, and
  - (d) Inserting at least one transcription termination DNA sequence of gram-negative origin in the downstream direction of said foreign gene encoding prokaryotic or eukaryotic polypeptides.
36. A process for the manufacture of pro- or eukaryotic polypeptide which process comprises transforming a host with an expression vector as claimed in any one of claims 20-30 containing the DNA sequence coding for said polypeptide operatively inserted into an expression control DNA sequence of claims 1 to 16, culturing the transformant under appropriate conditions of growth and isolating the derived polypeptide from the culture.

37. A process according to claim 36, wherein the vector is plasmidic shuttle vector capable of replication in gram-negative and gram-positive bacteria.
38. A process according to claim 37, wherein the plasmidic shuttle vector is capable of replication in a strain of *E. coli* and *Bacillus*.
39. A process according to claim 38, wherein the plasmidic shuttle vector is capable of replication in a strain of *E. coli* and *Bacillus subtilis*.
40. A process according to any one of claims 36 to 39, wherein the shuttle vector is p25RBSI DSM 3350.
41. A process according to any one of claims 36 to 39, wherein the shuttle vector is p25RBSII DSM 3351.
42. A process according to any one of claims 36 to 39, wherein the shuttle vector is p25RBSII DSM 3352.
43. A process according to any one of claims 36 to 39, wherein the shuttle vector is p602/18 DSM 3723.
44. A process according to any one of claims 36 to 39, wherein the shuttle vector is p602/19 DSM 3724.
45. A process according to any one of claims 36 to 39, wherein the shuttle vector is p602/20 DSM 3725.
46. A process according to any one of claims 36 to 39, wherein the shuttle vector is p602/21 DSM 3726.
47. A process according to claims 38 to 44, wherein the polypeptide is *E. coli* chloramphenicol acetyltransferase.
48. A process according to claims 45 to 46, wherein the polypeptide is mouse dihydrofolate reductase.
49. The use of an expression control DNA sequence of claims 1 to 16 for expressing a gene encoding pro- or eukaryotic polypeptides.

#### Revendications

1. Séquence d'ADN de contrôle d'expression de bactéries Gram positif comprenant, dans le sens aval de la transcription :
- (a) un promoteur de coliphage T5 ou T7;
  - (b) une séquence d'ADN synthétique codant pour un site de liaison ribosomique répondant à la formule

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5'                                     3'
AGCTTTATATAAGGAGGAGTTAAGCATGCAC   (SRBSI),
AATATATTCCTCCTCAATTCGTACGTGTCGA
3'                                     5'

5'                                     3'
AGCTTGGATTAAATTTAGGAGGAATTTAAGCATG (SRBSII),
ACCTAAATTTTAAATCCTCCTTAAATTC
3'                                     3'

5'                                     3'
AATTCATTAAAGAGGAGAAATTAAGTATGAGGG (RBSII, 3A+5A),
GTAATTTCTCCTCTTTAATTGATACTCCCTAG
3'                                     5'

5'                                     3'
AATTCATTAAAGAGGAGAAATTAAGTATGAGAG (RBSII) ou
GTAATTTCTCCTCTTTAATTGATACTCTCCTAG
3'                                     5'

5'                                     3'
AATTCATTAAAGAGGAGAAATTAAGTATGGAAG (RBSII, 9A);
GTAATTTCTCCTCTTTAATTGATACCTTCCTAG
3'                                     5'

```

(c) une séquence d'ADN de terminaison de transcription d'origine Gram négatif.

2. Séquence d'ADN de contrôle d'expression selon la revendication 1, dans laquelle ledit promoteur de coliphage T5 est le promoteur P<sub>N25</sub>.
3. Séquence d'ADN de contrôle d'expression selon la revendication 1, dans laquelle ledit promoteur de coliphage T5 est le promoteur P<sub>N28</sub>.
4. Séquence d'ADN de contrôle d'expression selon la revendication 1, dans laquelle ledit promoteur de coliphage T5 est le promoteur P<sub>G25</sub>.
5. Séquence d'ADN de contrôle d'expression selon la revendication 1, dans laquelle ledit promoteur de coliphage T5 est le promoteur P<sub>J5</sub>.
6. Séquence d'ADN de contrôle d'expression selon la revendication 1, dans laquelle ledit promoteur de coliphage T5 est le promoteur P<sub>D/E20</sub>.
7. Séquence d'ADN de contrôle d'expression selon la revendication 1, dans laquelle ledit promoteur de coliphage T5 est le promoteur P<sub>K28a</sub>.

8. Séquence d'ADN de contrôle d'expression selon la revendication 1, dans laquelle ledit promoteur de coliphage T5 est le promoteur P<sub>K285</sub>.
9. Séquence d'ADN de contrôle d'expression selon la revendication 1, dans laquelle ledit promoteur T7 est le promoteur T7A1.
10. Séquence d'ADN de contrôle d'expression selon la revendication 1, dans laquelle ledit promoteur T7 est le promoteur T7A2.
11. Séquence d'ADN de contrôle d'expression selon l'une quelconque des revendications 1 à 10, dans laquelle ladite séquence d'ADN de terminaison de transcription est le signal de terminaison t<sub>0</sub> de E. coli.
12. Séquence d'ADN de contrôle d'expression selon l'une quelconque des revendications 1 à 10, dans laquelle ladite séquence d'ADN de terminaison de transcription est le signal de terminaison T1 de E. coli.
13. Séquence d'ADN de contrôle d'expression selon l'une quelconque des revendications 1 à 10, dans laquelle ladite séquence d'ADN de terminaison de transcription est le signal de terminaison T2 de E. coli.
14. Séquence d'ADN de contrôle d'expression selon l'une quelconque des revendications 1 à 10, dans laquelle ladite séquence d'ADN de terminaison de transcription est le signal de terminaison T7 de E. coli.
15. Séquence d'ADN de contrôle d'expression selon l'une quelconque des revendications 1 à 14, capable de fonctionner dans Bacillus.
16. Séquence d'ADN de contrôle d'expression selon la revendication 15, capable de fonctionner dans Bacillus subtilis.
17. Séquence d'ADN synthétique codant pour un site de liaison ribosomique, capable de fonctionner dans des organismes Gram positif, répondant à la formule

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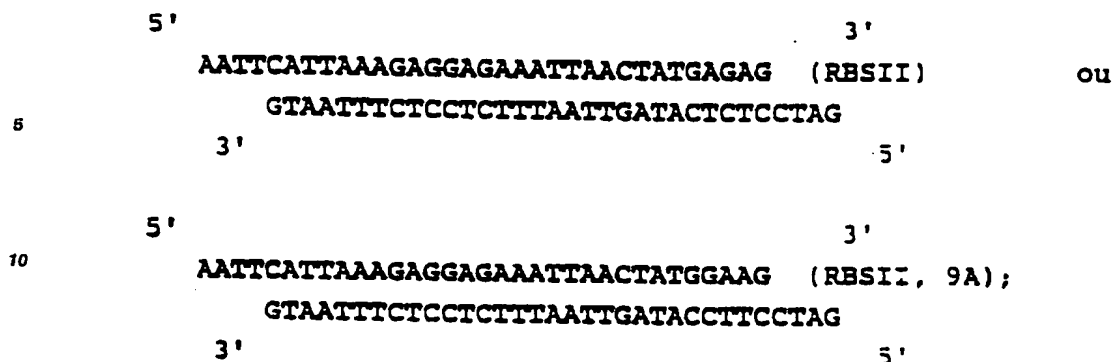
35      5'                                3'
      AGCTTTATATAAGGAGGAGTTAAGCATGCAC (SRBSI),
      AATATATTCCTCCTCAATTCGTACGTGTCGA
40      3'                                5'

      5'                                3'
45      AGCTTGGATTTAAAATTTAGGAGGAATTTAAGCATG (SRBSII),
      ACCTAAATTTTAAATCCTCCTTAAATTC
      3'                                3'

50      5'                                3'
      AATTCATTAAAGAGGAGAAATTAAGTATGAGGG (RBSII, 3A+5A),
      GTAATTTCTCCTCTTTAATTGATACTCCCCTAG
55      3'                                5'

```

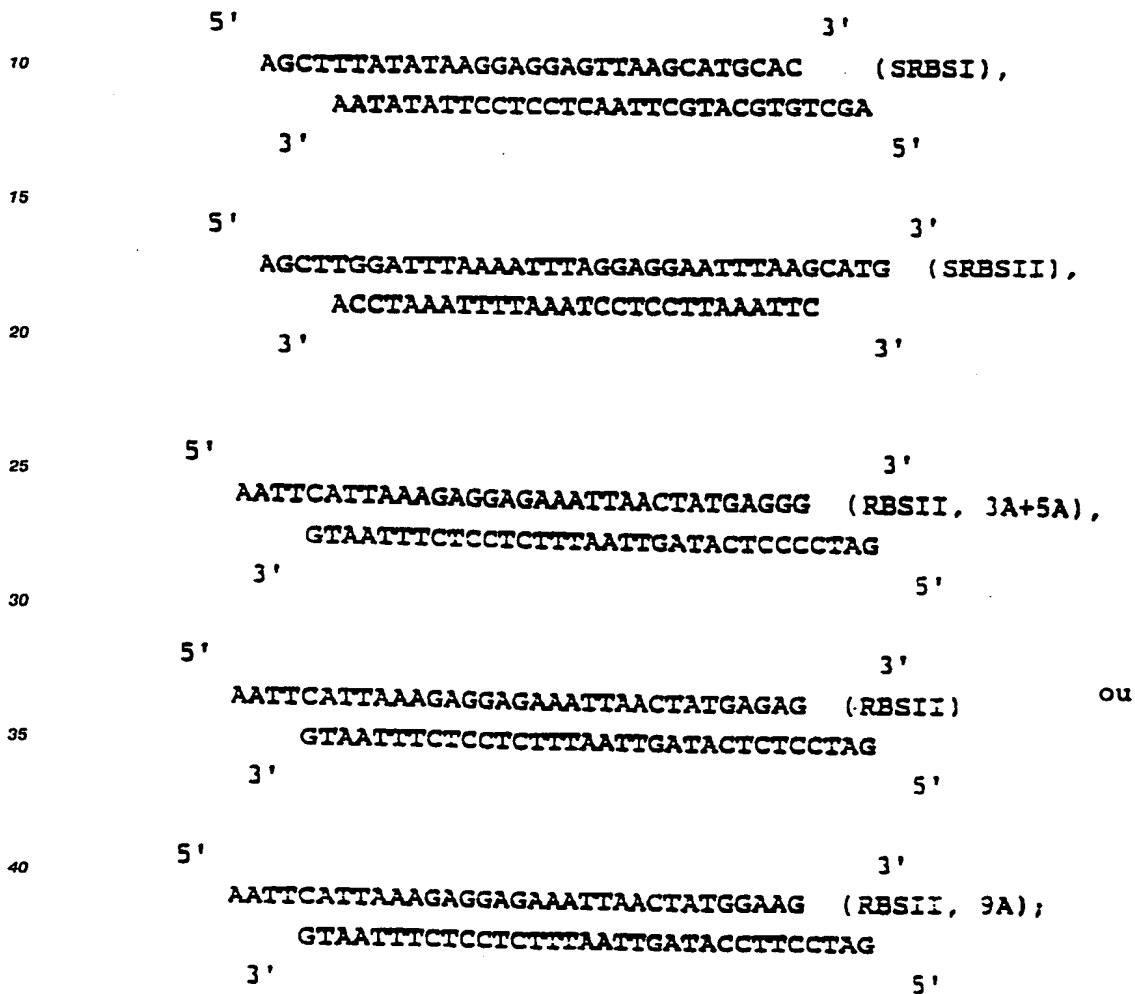




18. Séquence d'ADN synthétique codant pour un site de liaison ribosomique selon la revendication 17, capable de fonctionner dans *Bacillus*.
19. Séquence d'ADN synthétique codant pour un site de liaison ribosomique selon la revendication 18, capable de fonctionner dans *Bacillus subtilis*.
20. Vecteur d'expression contenant
  - (a) une séquence d'ADN de contrôle d'expression de bactéries Gram positif selon l'une quelconque des revendications 1 à 16, dans laquelle la séquence d'ADN codant pour un site de liaison ribosomique est éventuellement liée de façon opérante à un gène étranger codant pour des polypeptides procaryotes ou encaryotes,
  - (b) au moins un vecteur origine de réplication et
  - (c) au moins un gène de résistance à un antibiotique.
21. Vecteur d'expression selon la revendication 20, qui est un vecteur navette plasmidique capable de réplication dans les bactéries Gram négatif et Gram positif.
22. Vecteur d'expression selon la revendication 21, qui est capable de réplication dans une souche de *E. coli* et de *Bacillus*.
23. Vecteur d'expression selon la revendication 22, qui est capable de réplication dans une souche de *E. coli* et de *B. subtilis*.
24. Vecteur d'expression selon l'une quelconque des revendications 20 à 23, qui est p25RBSI DSM 3350.
25. Vecteur d'expression selon l'une quelconque des revendications 20 à 23, qui est p25RBSII DSM 3351.
26. Vecteur d'expression selon l'une quelconque des revendications 20 à 23, qui est p25RBSII DSM 3352.
27. Vecteur d'expression selon l'une quelconque des revendications 20 à 23, qui est p602/18 DSM 3723.
28. Vecteur d'expression selon l'une quelconque des revendications 20 à 23, qui est p602/19 DSM 3724.
29. Vecteur d'expression selon l'une quelconque des revendications 20 à 23, qui est p602/20 DSM 3725.
30. Vecteur d'expression selon l'une quelconque des revendications 20 à 23, qui est p602/21 DSM 3726.
31. Transformant portant un vecteur d'expression selon l'une quelconque des revendications 20 à 30.
32. Transformant selon la revendication 31, qui est une souche de *Bacillus subtilis*.
33. Transformant selon la revendication 32, qui est une souche BR 151 de *Bacillus subtilis*.

34. Procédé de fabrication d'une séquence d'ADN de contrôle d'expression de bactéries Gram positif selon l'une quelconque des revendications 1 à 16, ce procédé comprenant la combinaison, dans le sens aval de la transcription :

- (a) d'un promoteur de coliphage T5 ou T7;  
 5 (b) d'une séquence d'ADN synthétique codant pour un site de liaison ribosomique répondant à la formule



et

- (c) d'une séquence d'ADN de terminaison de transcription d'origine Gram négatif avec une unité fonctionnelle par des techniques de recombinaison d'ADN bien connues dans le domaine.

35. Procédé de fabrication d'un vecteur d'expression selon l'une quelconque des revendications 20 à 30, par des techniques de recombinaison d'ADN bien connues dans le domaine, comprenant les étapes qui consistent à :

- (a) insérer dans un vecteur de clonage existant, dans le sens aval de la transcription, au moins un promoteur de coliphage T5 ou T7 et une séquence d'ADN codant pour un site de liaison ribosomique telle que définie dans la revendication 17,  
 55 (b) prévoir dans ledit vecteur de clonage au moins un site d'endonuclease de restriction près de ladite séquence d'ADN codant pour un site de liaison ribosomique,

- (c) insérer au moins un gène étranger codant pour des polypeptides procaryotes ou eucaryotes dans ledit site d'endonucléase de restriction près de ladite séquence d'ADN codant pour un site de liaison ribosomique, et
- (d) insérer au moins une séquence d'ADN de terminaison de transcription d'origine Gram négatif dans le sens aval dudit gène étranger codant pour des polypeptides procaryotes ou eucaryotes.
36. Procédé de fabrication d'un polypeptide procaryote ou eucaryote, ce procédé comprenant la transformation d'une hôte avec un vecteur d'expression selon l'une quelconque des revendications 20-30 contenant la séquence d'ADN codant pour ledit polypeptide inséré de façon opérante dans une séquence d'ADN de contrôle d'expression des revendications 1 à 16, la culture du transformant dans des conditions de croissance appropriées et l'isolement de la culture du polypeptide dérivé.
37. Procédé selon la revendication 36, dans lequel le vecteur est un vecteur navette plasmidique capable de répllication dans des bactéries Gram négatif et Gram positif.
38. Procédé selon la revendication 37, dans lequel le vecteur navette plasmidique est capable de répllication dans une souche de *E. coli* et de *Bacillus*.
39. Procédé selon la revendication 38, dans lequel le vecteur navette plasmidique est capable de répllication dans une souche de *E. coli* et de *Bacillus subtilis*.
40. Procédé selon l'une quelconque des revendications 36 à 39, dans lequel le vecteur navette est p25RBSI DSM 3350.
41. Procédé selon l'une quelconque des revendications 36 à 39, dans lequel le vecteur navette est p25RBSII DSM 3351.
42. Procédé selon l'une quelconque des revendications 36 à 39, dans lequel le vecteur navette est p25RBSII DSM 3352.
43. Procédé selon l'une quelconque des revendications 36 à 39, dans lequel le vecteur navette est p602/18 DSM 3723.
44. Procédé selon l'une quelconque des revendications 36 à 39, dans lequel le vecteur navette est p602/19 DSM 3724.
45. Procédé selon l'une quelconque des revendications 36 à 39, dans lequel le vecteur navette est p602/20 DSM 3725.
46. Procédé selon l'une quelconque des revendications 36 à 39, dans lequel le vecteur navette est p602/21 DSM 3726.
47. Procédé selon les revendications 36 à 44, dans lequel le polypeptide est la chloramphénicol-acétyltransférase de *E. coli*.
48. Procédé selon les revendications 45 à 46, dans lequel le polypeptide est la dihydrofoliate-réductase de souris.
49. Utilisation d'une séquence d'ADN de contrôle d'expression des revendications 1 à 16 pour exprimer un gène codant pour des polypeptides procaryotes ou eucaryotes.

#### Ansprüche

1. Eine gram-positive, bakteriell Expr ssionskontroll-DNA-Sequenz, nthaltend in Transkriptionsrichtung
- (a) einen Coliphag n T5 oder T7 Promotor;
- (b) ein synthetische DNA-Sequenz, die eine Ribosomen-Bindungsstelle kodiert, mit der Formel

5' 3'  
 AGCTTTATATAAGGAGGAGTTAAGCATGCAC (SRBSI),  
 AATATATTCCTCCTCAATTCGTACGTGTCGA  
 5 3' 5'

5' 3'  
 10 AGCTTGGATT TAAAAATTTAGGAGGAATTTAAGCATG (SRBSII),  
 ACCTAAATTTTAAATCCTCCTTAAATTC  
 3' 3'

15 5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGAGGG (RBSII, 3A+5A),  
 GTAATTTCTCCTCTTTAATTGATACTCCCCTAG  
 20 3' 5'

25 5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGAGAG (RBSII) oder  
 GTAATTTCTCCTCTTTAATTGATACTCTCCTAG  
 3' 5'

30 5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGGAAG (RBSII, 9A);  
 GTAATTTCTCCTCTTTAATTGATACCTTCCTAG  
 35 3' 5'

und

(c) eine DNA-Sequenz für die Transkriptionstermination gram-negativen Ursprungs.

- 40
2. Eine Expressionskontroll-DNA-Sequenz gemäss Anspruch 1, worin besagter Coliphage T5 Promotor der P<sub>N25</sub> Promotor ist.
  - 45 3. Eine Expressionskontroll-DNA-Sequenz gemäss Anspruch 1, worin besagter Coliphage T5 Promotor der P<sub>N26</sub> Promotor ist.
  4. Eine Expressionskontroll-DNA-Sequenz gemäss Anspruch 1, worin besagter Coliphage T5 Promotor der P<sub>G25</sub> Promotor ist.
  - 50 5. Eine Expressionskontroll-DNA-Sequenz gemäss Anspruch 1, worin besagter Coliphage T5 Promotor der P<sub>J5</sub> Promotor ist.
  6. Eine Expressionskontroll-DNA-Sequenz gemäss Anspruch 1, worin besagter Coliphage T5 Promotor der P<sub>D/E20</sub> Promotor ist.
  - 55 7. Eine Expressionskontroll-DNA-Sequenz gemäss Anspruch 1, worin besagter Coliphage T5 Promotor der P<sub>K28a</sub> Promotor ist.

8. Eine Expressionskontroll-DNA-Sequenz gemäss Anspruch 1, worin besagter Coliphage T5 Promotor der  $P_{K285}$  Promotor ist.
- 5 9. Eine Expressionskontroll-DNA-Sequenz gemäss Anspruch 1, worin besagter Coliphage T7 Promotor der T7A1 Promotor ist.
10. Eine Expressionskontroll-DNA-Sequenz gemäss Anspruch 1, worin besagter Coliphage T7 Promotor der T7A2 Promotor ist.
- 10 11. Eine Expressionskontroll-DNA-Sequenz gemäss einem der Ansprüche 1 bis 10, worin besagte DNA-Sequenz für die Transkriptionstermination der E. coli  $t_0$  Terminator ist.
12. Eine Expressionskontroll-DNA-Sequenz gemäss einem der Ansprüche 1 bis 10, worin besagte DNA-Sequenz für die Transkriptionstermination der E. coli T1 Terminator ist.
- 15 13. Eine Expressionskontroll-DNA-Sequenz gemäss einem der Ansprüche 1 bis 10, worin besagte DNA-Sequenz für die Transkriptionstermination der E. coli T2 Terminator ist.
14. Eine Expressionskontroll-DNA-Sequenz gemäss einem der Ansprüche 1 bis 10, worin besagte DNA-Sequenz für die Transkriptionstermination der E. coli T7 Terminator ist.
- 20 15. Eine Expressionskontroll-DNA-Sequenz gemäss einem der Ansprüche 1 bis 14, die in Bacillus funktionsfähig ist.
- 25 16. Eine Expressionskontroll-DNA-Sequenz gemäss Anspruch 15, die in Bacillus subtilis funktionsfähig ist.
17. Eine synthetische DNA-Sequenz, die eine Ribosomen-Bindungsstelle kodiert, welche in gram-positiven Organismen funktionsfähig ist, mit der Formel

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5' 3'  
 AGCTTTATATAAGGAGGAGTTAAGCATGCAC (SRBSI),  
 5 AATATATTCCTCCTCAATTCGTACGTGTCGA  
 3' 5'  
 5' 3'  
 10 AGCTTGGATTATAAAATTTAGGAGGAATTTAAGCATG (SRBSII),  
 ACCTAAATTTTAAATCCTCCTTAAATTC  
 3' 3'  
 15 5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGAGGG (RBSII, 3A+5A),  
 20 GTAATTTCTCCTCTTTAATTGATACTCCCCTAG  
 3' 5'  
 25 5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGAGAG (RBSII) oder  
 GTAATTTCTCCTCTTTAATTGATACTCTCCTAG  
 3' 5'  
 30 5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGGAAG (RBSII, 5A);  
 35 GTAATTTCTCCTCTTTAATTGATACCTTCCTAG  
 3' 5'

- 40 18. Eine eine Ribosomen-Bindungsstelle kodierende synthetische DNA-Sequenz gemäss Anspruch 17, die in *Bacillus* funktionsfähig ist.
19. Eine eine Ribosomen-Bindungsstelle kodierende synthetische DNA-Sequenz gemäss Anspruch 18, die in *Bacillus subtilis* funktionsfähig ist.
- 45 20. Ein Expressionsvektor mit  
 (a) einer gram-positiven bakteriellen Expressionskontroll-DNA-Sequenz nach einem der Ansprüche 1 bis 18, worin besagte DNA-Sequenz, die eine Ribosomen-Bindungsstelle kodiert, wahlweise operativ mit einem Fremdgen, welches prokaryote oder eukaryote Polypeptide kodiert, verknüpft ist,  
 50 (b) wenigstens einem von einem Vektor stammenden Replikationsstartpunkt und  
 (c) wenigstens einem Antibiotikaresistenz-Gen.
21. Ein Expressionsvektor gemäss Anspruch 20, der ein bifunktionaler Plasmid-Vektor ist, der in gram-negativen und gram-positiven Bakterien replizieren kann.
- 55 22. Ein Expressionsvektor gemäss Anspruch 21, der in einem *E. coli* Stamm und in *Bacillus* replizieren kann.

23. Ein Expressionsvektor gemäss Anspruch 22, der in einem E. coli Stamm und in B. subtilis replizieren kann.
24. Ein Expressionsvektor gemäss einem der Ansprüche 20 bis 23 mit der Bezeichnung p25RBSI DSM 3350.
25. Ein Expressionsvektor gemäss einem der Ansprüche 20 bis 23 mit der Bezeichnung p25RBSII DSM 3351.
26. Ein Expressionsvektor gemäss einem der Ansprüche 20 bis 23 mit der Bezeichnung p25<sup>+</sup>RBSII DSM 3352.
27. Ein Expressionsvektor gemäss einem der Ansprüche 20 bis 23 mit der Bezeichnung p602/18 DSM 3723.
28. Ein Expressionsvektor gemäss einem der Ansprüche 20 bis 23 mit der Bezeichnung p602/19 DSM 3724.
29. Ein Expressionsvektor gemäss einem der Ansprüche 20 bis 23 mit der Bezeichnung p602/20 DSM 3725.
30. Ein Expressionsvektor gemäss einem der Ansprüche 20 bis 23 mit der Bezeichnung p602/21 DSM 3726.
31. Eine Transformante, die einen Expressionsvektor gemäss einem der Ansprüche 20 bis 30 trägt.
32. Eine Transformante gemäss Anspruch 31, die ein Bacillus subtilis Stamm ist.
33. Eine Transformante gemäss Anspruch 32, die ein Bacillus subtilis BR 151 Stamm ist.
34. Ein Verfahren zur Herstellung einer gram-positiven bakteriellen Expressionskontroll-DNA-Sequenz gemäss einem der Ansprüche 1 bis 16, dadurch gekennzeichnet, dass man in Transkriptionsrichtung  
 (a) einen Coliphagen T5 oder T7 Promotor;  
 (b) eine synthetische DNA-Sequenz, die eine Ribosomen-Bindungsstelle kodiert, mit der Formel

```

5'                                     3'
AGCTTTATATAAGGAGGAGTTAAGCATGCAC   (SRBSI),
AATATATTCCTCCTCAATTCGTACGTGTCGA
3'                                     5'

```

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5'                                     3'
AGCTTGGATTTTAAAATTTAGGAGGAATTTAAGCATG   (SRBSII),
ACCTAAATTTTAAATCCTCCTTAAATTC
3'                                     3'

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5'                                     3'
AATTCATTAAAGAGGAGAAATTAAGTATGAGGG   (RBSII, 3A+5A),
GTAATTTCTCCTCTTTAATTGATACTCCCTAG
3'                                     5'

```

5' 3'  
 AATTCATTAAAGAGGAGAAATTAAGTATGAGAG (RBSII) oder  
 5 3' GTAAATTCCTCCTCTTTAATTGATACTCTCCTAG 5'  
 5' 3'  
 10 AATTCATTAAAGAGGAGAAATTAAGTATGGAAG (RBSII, 9A);  
 3' 5' GTAAATTCCTCCTCTTTAATTGATACCTTCCTAG  
 15 und

- (c) eine DNA-Sequenz für die Transkriptionstermination gram-negativen Ursprungs mittels bekannten DNA-Rekombinationstechniken zu einer funktionellen Einheit verbindet.
- 20 35. Ein Verfahren zur Herstellung eines Expressionsvektors gemäss einem der Ansprüche 20 bis 30 mittels bekannten DNA-Rekombinationstechniken, dadurch gekennzeichnet, dass man
- 25 (a) in einen Klonierungsvektor in Transkriptionsrichtung wenigstens einen Coliphagen T5 oder T7 Promotor und eine gemäss Anspruch 17 definierte DNA-Sequenz, die eine Ribosomen-Bindungsstelle kodiert, einbaut,
- (b) genannten Klonierungsvektor mit wenigstens einem Restriktionsendonucleaseort neben genannter DNA-Sequenz, die eine Ribosomen-Bindungsstelle kodiert, versieht,
- (c) wenigstens ein Fremdgen, welches prokaryote oder eukaryote Polypeptide kodiert, in besagten Restriktionsendonucleaseort neben genannter Sequenz, die eine Ribosomen-Bindungsstelle kodiert, einbaut, und
- 30 (d) wenigstens eine DNA-Sequenz für die Transkriptionstermination gram-negativen Ursprungs flussabwärts von genanntem Fremdgen, welches prokaryote oder eukaryote Polypeptide kodiert, einbaut.
- 35 36. Ein Verfahren zur Herstellung eines pro- oder eukaryoten Polypeptids, dadurch gekennzeichnet, dass man einen Wirtsorganismus mit einem Expressionsvektor gemäss einem der Ansprüche 20-30, welcher die für genanntes Polypeptid kodierende DNA-Sequenz operativ in eine Expressionskontroll-DNA-Sequenz der Ansprüche 1 bis 16 eingebaut enthält, transformiert, die Transformante unter geeigneten Wachstumsbedingungen züchtet und das erhaltene Polypeptid aus der Kultur isoliert.
- 40 37. Ein Verfahren gemäss Anspruch 36, worin der Vektor ein bifunktionaler Plasmid-Vektor ist, der in gram-negativen und gram-positiven Bakterien replizieren kann.
- 45 38. Ein Verfahren gemäss Anspruch 37, worin der bifunktionale Plasmid-Vektor in einem E. coli Stamm und in Bacillus replizieren kann.
39. Ein Verfahren gemäss Anspruch 38, worin der bifunktionale Plasmid-Vektor in einem E. coli Stamm und in Bacillus subtilis replizieren kann.
- 50 40. Ein Verfahren gemäss einem der Ansprüche 36 bis 39, worin der bifunktionale Vektor p25RBSI DSM 3350 ist.
41. Ein Verfahren gemäss einem der Ansprüche 36 bis 39, worin der bifunktionale Vektor p25RBSII DSM 3351 ist.
- 55 42. Ein Verfahren gemäss einem der Ansprüche 36 bis 39, worin der bifunktionale Vektor p25<sup>\*</sup>RBSII DSM 3352 ist.



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43. Ein Verfahren gemäss einem der Ansprüche 36 bis 39, worin der bifunktionale Vektor p602/18 DSM 3723 ist.
- 5 44. Ein Verfahren gemäss einem der Ansprüche 36 bis 39, worin der bifunktionale Vektor p602/19 DSM 3724 ist.
45. Ein Verfahren gemäss einem der Ansprüche 36 bis 39, worin der bifunktionale Vektor p602/20 DSM 3725 ist.
- 10 46. Ein Verfahren gemäss einem der Ansprüche 36 bis 39, worin der bifunktionale Vektor p602/21 DSM 3726 ist.
47. Ein Verfahren gemäss einem der Ansprüche 36 bis 44, worin das Polypeptid E. coli Chloramphenicol-Acetyltransferase ist.
- 15 48. Ein Verfahren gemäss einem der Ansprüche 45 bis 46, worin das Polypeptid Dihydrofolatreduktase der Maus ist.
- 20 49. Die Verwendung einer Expressionskontroll-DNA-Sequenzen nach den Ansprüchen 1 bis 16 zur Expression eines Gens, welches pro- oder eukaryote Polypeptide kodiert.

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Fig.1

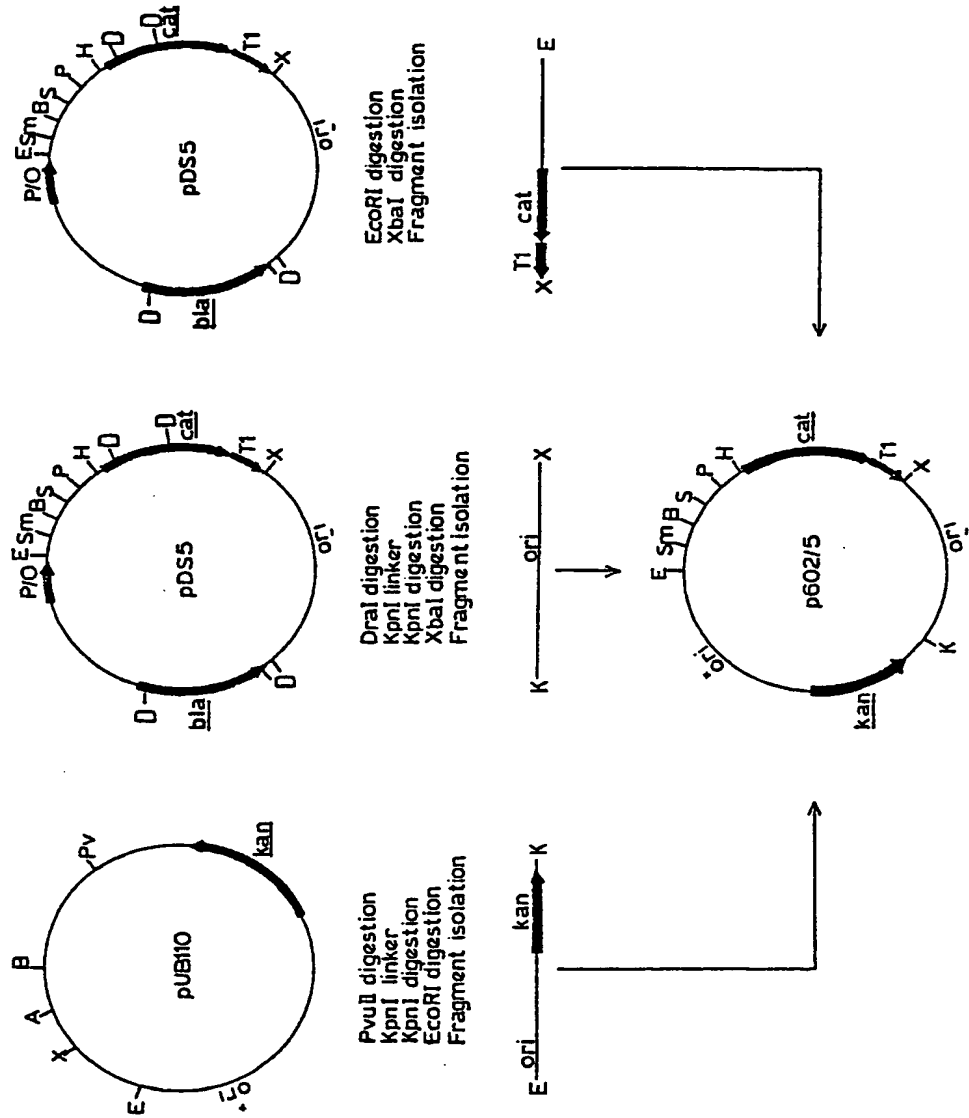


Fig.2

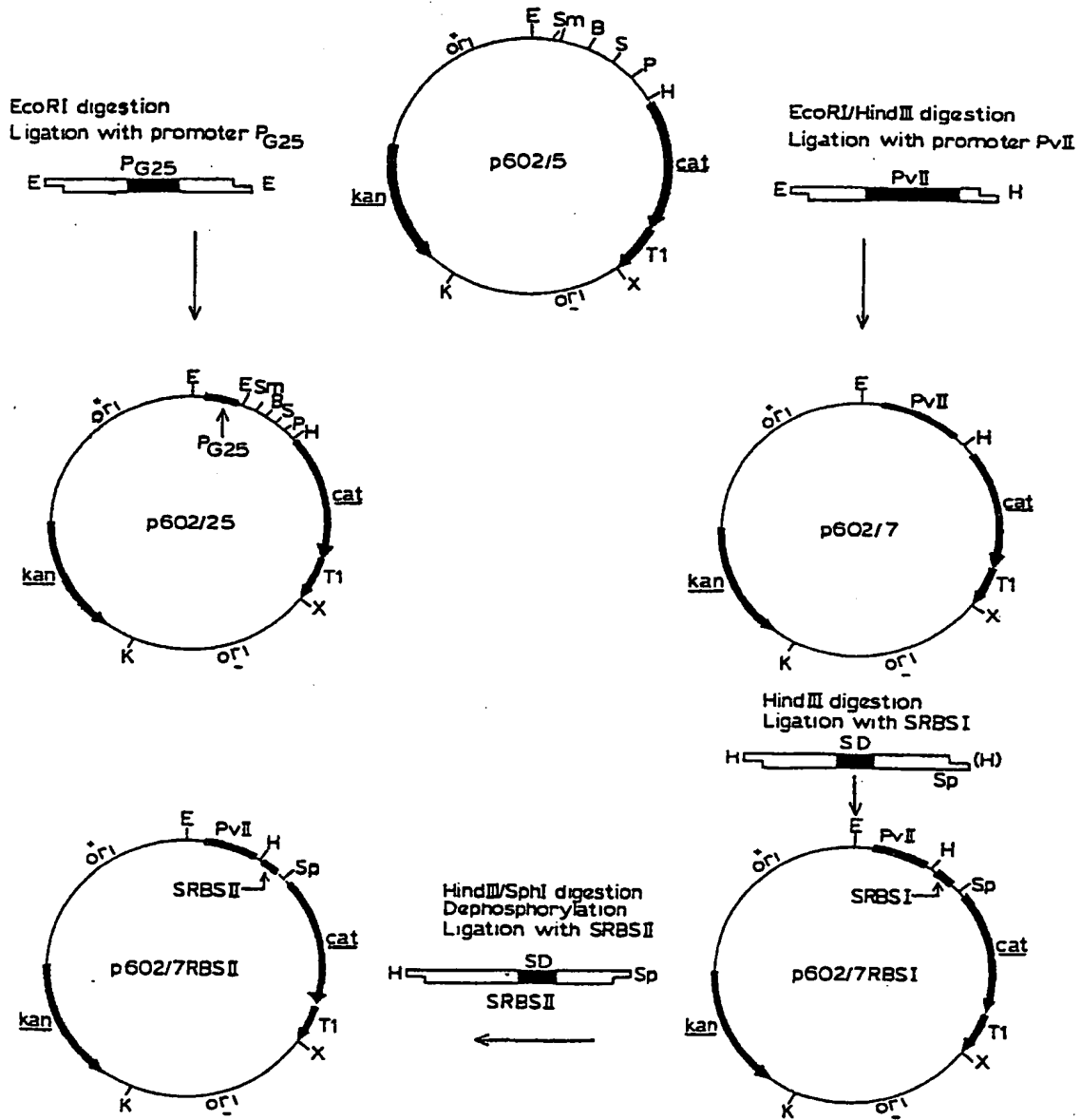


Fig.3

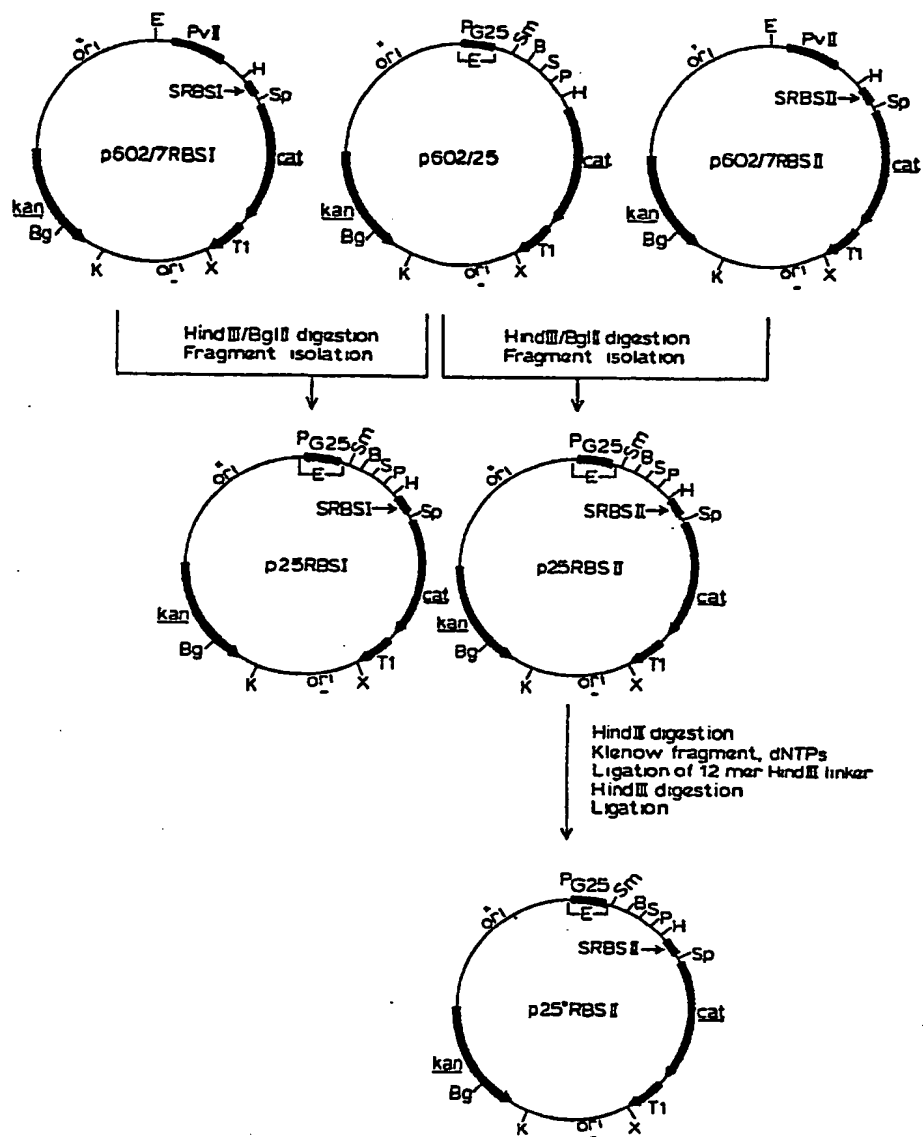


Fig.4

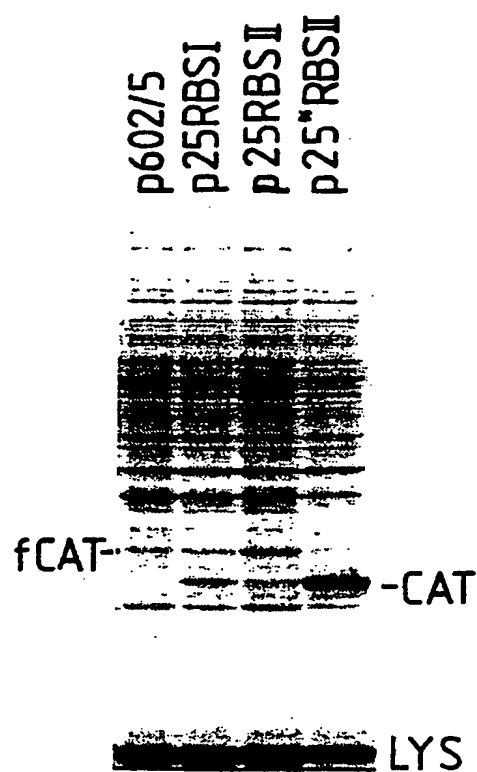


Fig.5

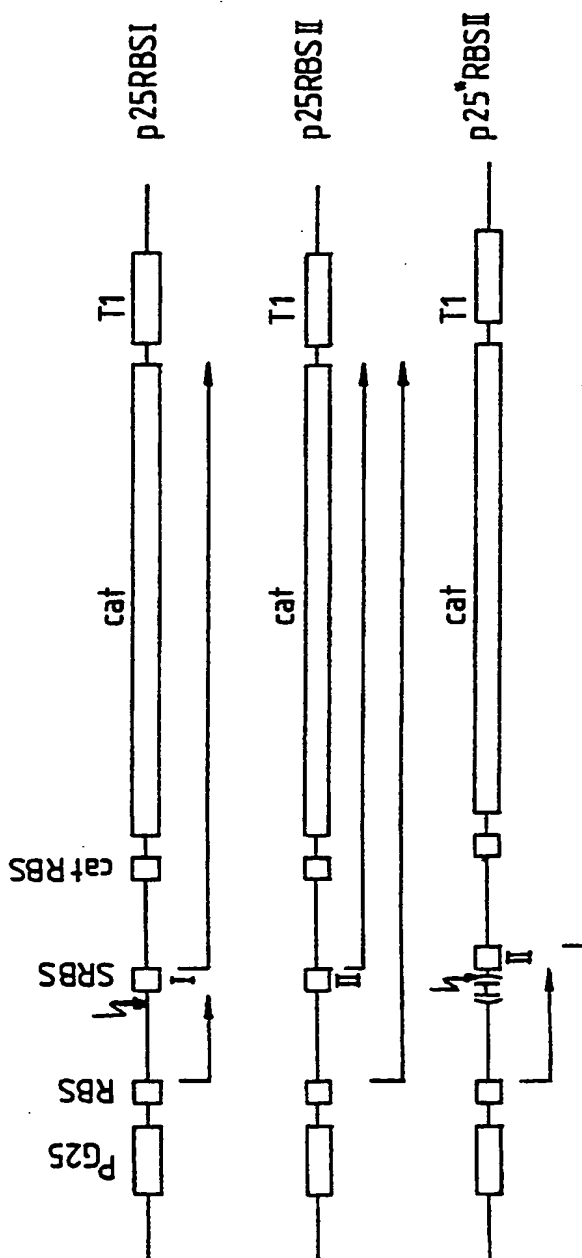


Fig.6

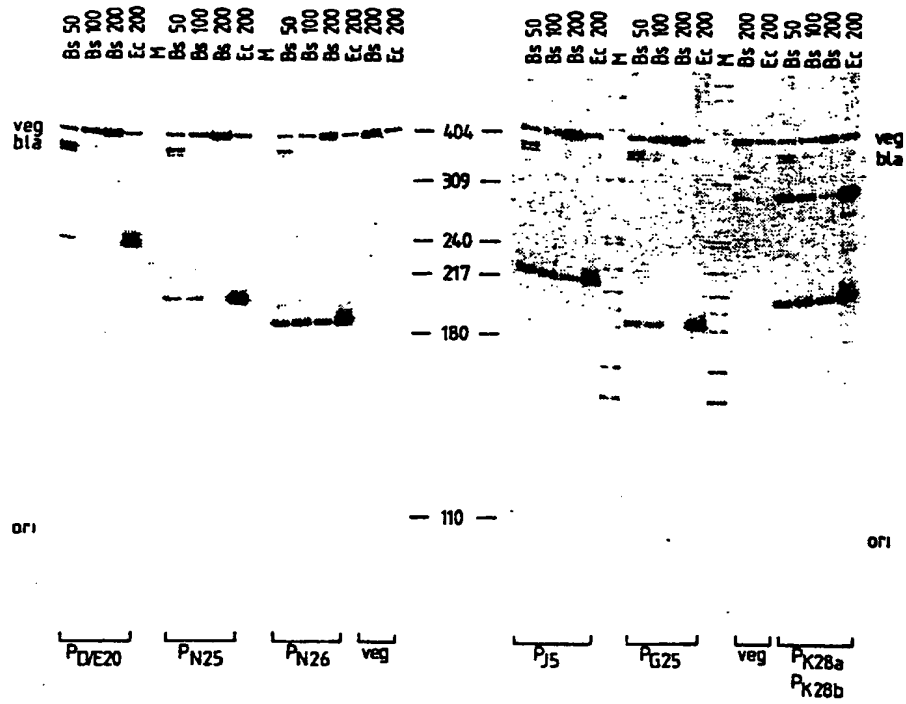


Fig. 7

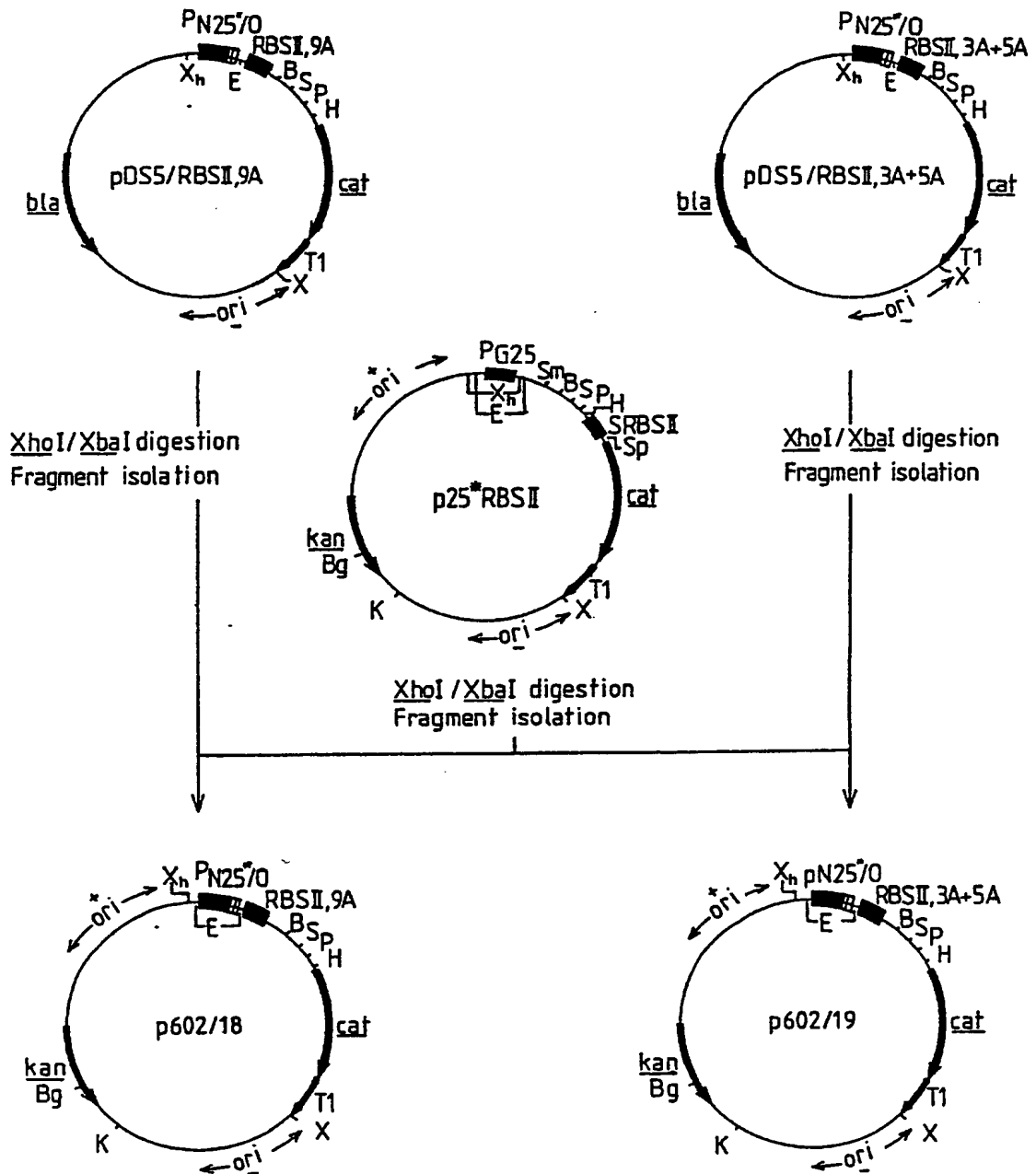




Fig. 8

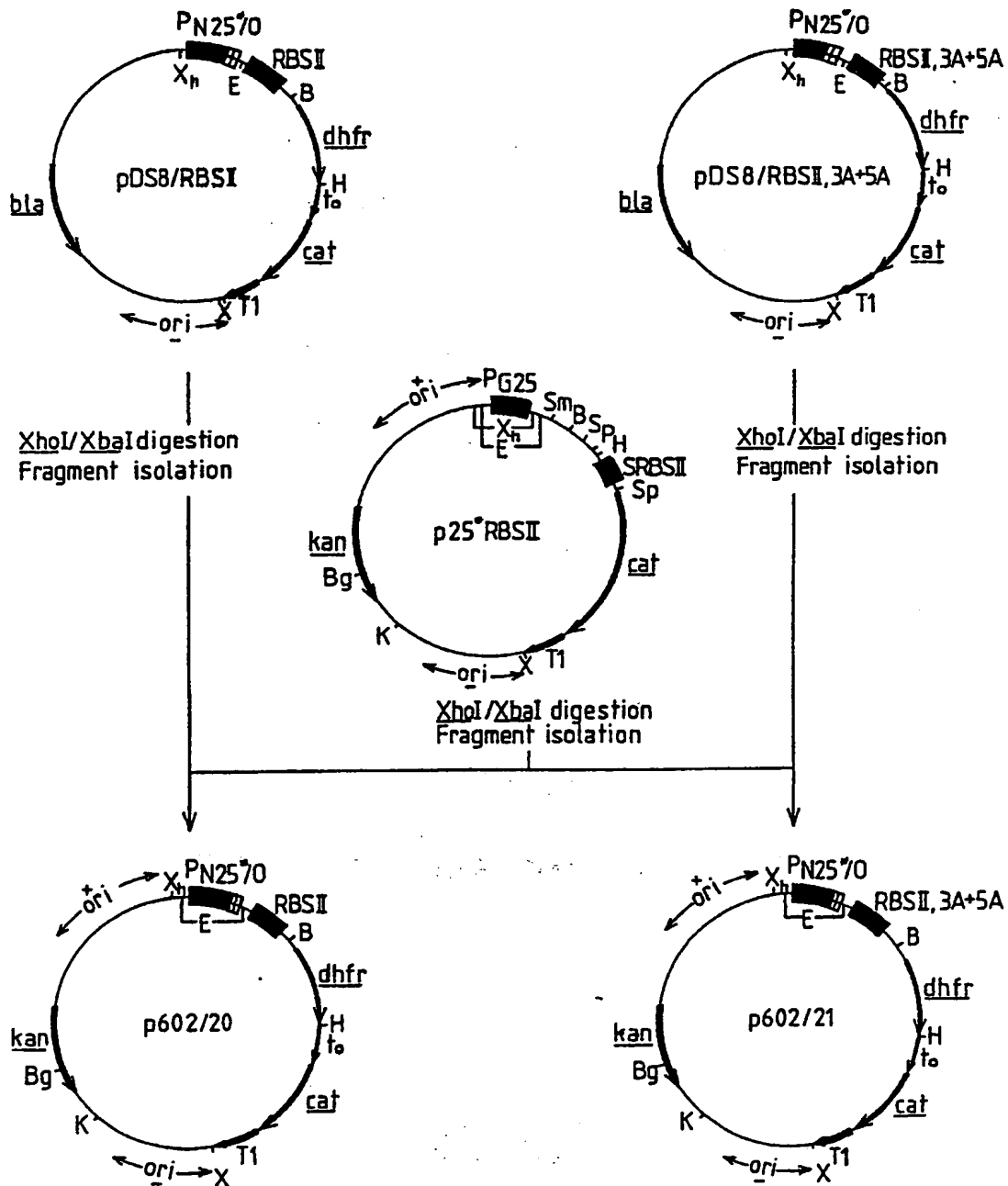


Fig. 9

